The cloning of genes involved in carnitine-dependent activities in *Saccharomyces cerevisiae*

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

L-Carnitine is a unique and important compound in eukaryotic cells. In *Saccharomyces cerevisiae*, L-carnitine plays a role in the transfer of acetyl groups from the peroxisomes to the mitochondria. This takes place with the help of the carnitine acetylcarnitine shuttle. The activated acyl group of acetyl-CoA in the peroxisome is transferred to carnitine with the help of a peroxisomal carnitine acetyltransferase to form an acetylcarnitine ester, releasing the CoA-SH. This ester is then transported through the peroxisomal membrane to the cytosol from where it is transported to the mitochondrion. After transport of the acetylcarnitine through the mitochondrial membranes, the reverse reaction takes place in the matrix with the help of a mitochondrial carnitine acetyltransferase, releasing carnitine and the acyl group. In *S. cerevisiae*, the main carnitine acetyltransferase contributing to >95% of the total carnitine acetyltransferase activity, is encoded by a single gene, *CAT2*. Cat2p has a peroxisomal and mitochondrial targeting signal and is located to the peroxisomal membrane and the inner-mitochondrial membrane, respectively.

The reason for the activated acyl group to be transferred in the form of an acetylcarnitine, is that the peroxisomal membrane is impermeable to acetyl-CoA. This means that the acyl group needs to be transported in the form of intermediate compounds. Acetyl-CoA is formed in the peroxisome of *S. cerevisiae* as a result of β-oxidation of fatty acids. In yeast, the peroxisome is the sole site for β-oxidation. Fatty acids are transported to the peroxisome where they are oxidized by the β-oxidation cycle to form two-carbon acyl groups in the form of acetyl-CoA. These two-carbon acyl groups are then transferred from the peroxisome to the rest of the cell for gluconeogenesis and other anabolic pathways, or used in the tricarboxylic acid cycle (TCA) of the mitochondia to generate ATP. In this way, it is possible for the cell to use fatty acid as a sole carbon source.

There is a second pathway allowing for the utilization of activated acyl groups produced in the peroxisome and that is the glyoxylate cycle. The glyoxylate cycle is a modified TCA cycle, which results in the synthesis of C_4 succinate from two molecules of acetyl-CoA. In *S. cerevisiae*, all of the enzymes of the glyoxylate cycle are located in the peroxisome except for one, whereas in other yeasts studied, all of the glyoxylate enzymes are peroxisomal. As a result of the glyoxylate cycle, the two carbons of acetyl-CoA can leave the peroxisome in the form of succinate or other TCA intermediates like malate and citrate. These compounds are transferred through dicarboxylic acid carriers present in the peroxisomal membrane and used in further metabolic needs of the cell.

To understand the role of carnitine in the cell, a strategy for the cloning of genes involved in carnitine-dependent activities in *S. cerevisiae* was developed. The disruption of the citrate synthetase gene, *CIT2*, of the glyoxylate cycle yielded a strain that was dependent

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on carnitine when grown on the fatty acid oleic acid. This allowed for a mutagenesis strategy based on negative selection of mutants affected in carnitine-dependent activities. The Δ*cit2* strain was mutagenized and plated on minimal media. After replica plating on oleic acid media, mutant strains were selected that were unable to grow even in the presence of carnitine. In order to eliminate strains with defects in peroxisome biogenesis and β-oxidation, and only select for strains with defects in carnitine-dependent activities, the mutant strains were transformed with the *CIT2* gene to restore the glyoxylate cycle. Mutants that grew on oleic acid after transformation, and which are therefore not affected in activities independent of carnitine, were retained for further analysis. Transforming one of these mutants with a *S. cerevisiae* genomic library for functional complementation, yielded a clone carrying the *YAT1* gene, coding for the carnitine acetyltransferase of the outer-mitochondrial membrane. No phenotype had previously been assigned to a mutant allele of this gene.

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OPSOMMING

L-Karnitien is 'n unieke en belangrike verbinding in eukariotiese selle. In *Saccharomyces cerevisiae* speel L-karnitien 'n rol in die oordrag van asielgroepe van die peroksisoom na die mitochondrion. Dit vind plaas met behulp van die karnitien-asetielkarnitien-weg. Die geaktiveerde asiel groep van asetiel-KoA in die peroksisoom word na karnitien oorgedra met behulp van 'n peroksisomale karnitien-asetielkarnitien-transferase-ensiem om 'n asetielkarnitien ester te vorm, waarna die KoA-SH vrygestel word. Hierdie ester word dan deur die peroksisomale membraan na die sitoplasma vervoer waarna dit na die mitochondrion vervoer word. Nadat die asetielkarnitien deur die mitochondriale membrane vervoer is, vind die omgekeerde reaksie in die matriks plaas met behulp van die mitochondriale karnitien-asetielkarnitien-transferase-ensiem, waarna die karnitien en die asielgroep vrygestel word. In *S. cerevisiae* word die hoof karnitien-asetielkarnitien transferase wat tot >95% van die totale karnitien-asetielkarnitien-transferase-aktiwiteit bydra, deur 'n enkele geen, *CAT2* gekodeer. CAT2p het 'n peroksisomale en mitochondriale teikensein en dit word onderskeidelik na die peroksisomale en binne-mitochondriale membrane gelokaliseer.

Die geaktiveerde asielgroep word in die vorm van 'n asetielkarnitien vervoer omdat die peroksisomale membraan ondeurlaatbaar vir asetiel-KoA is. Dit beteken dat die asielgroepe slegs in die vorm van intermediêre verbindings vervoer kan word. Asetiel-KoA word weens β-oksidasie van vetsure in die peroksisoom van *S. cerevisiae* gevorm. In gis is die peroksisoom die enigste plek waar β-oksidasie plaasvind. Vetsure word na die peroksisoom vervoer waar dit deur die β-oksidasiesiklus geoksideer word om twee-koolstof asielgroepe in die vorm van asetiel-KoA te vorm. Hierdie twee-koolstof asielgroepe word dan vanaf die peroksisoom na die res van die sel vervoer vir glukoneogenese en ander metaboliese paaie, of dit word in die trikarboksielsuursiklus (TKS) van die mitochondrion gebruik om ATP te genereer. Op hierdie wyse is dit moontlik vir die sel om vetsure as enigste koolstofbron te benut.

Die glioksilaatsiklus is 'n tweede weg wat die benutting van asielgroepe, wat in die peroksisoom geproduseer is, toelaat. Die glioksilaatsiklus is 'n gemodifiseerde TKS-siklus wat die sintese van C_4 suksinaat van uit twee molekules asetiel-KoA bewerkstellig. In teenstelling met ander giste waar al die glioksilaatsiklus ensieme in die peroksisoom geleë is, kom een van *S. cerevisiae* se ensieme buite die peroksisoom voor. Die resultaat van die glioksilaatsiklus is dat die twee koolstowwe van asetiel-KoA die peroksisoom in die vorm van suksinaat of ander TKS-intermediêre verbindings soos malaat en sitraat, kan verlaat. Hierdie verbindings word deur middel van dikarboksielsuur-transporters in die peroksisomale membraan vervoer en word dan vir verdere metaboliese behoeftes in die sel gebruik.

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Om die rol van karnitien in die sel te verstaan, is 'n strategie ontwikkel om gene wat by karnitien-afhanklike aktiwiteite in *S. cerevisiae* betrokke is, te kloneer. Die disrupsie van die sitraatsintesegeen, *CIT2*, van die glioksilaatsiklus het 'n ras gelewer wat van karnitien vir groei op die vetsuur oleiensuur afhanklik was. Die Δ*cit2*-ras is gemuteer en op minimale media uitgeplaat. Na replika-platering op oleiensuur media, is mutante rasse geselekteer wat nie gegroei het nie, selfs nie in die teenwoordigheid van karnitien nie. Om mutantrasse uit te skakel wat defekte in peroksisoom-biogenese en β-oksidasie het en net mutantrasse te selekteer wat defekte in karnitien-afhanklike aktiwiteite het, is die rasse met die *CIT2*-geen getransformeer om die glioksilaatsiklus te herstel. Mutante wat na transformasie op oleiensuur gegroei het, en dus nie in aktiwiteite onafhanklik van karnitien geaffekteer is nie, is behou en aan verdere analise blootgestel. Komplimentering van een van hierdie mutante met 'n *S. cerevisiae* genomiese biblioteek, het 'n kloon wat die geen *YAT1* bevat, gelewer. *YAT1* is 'n geen wat die karnitienasetieltransferase van die buite-mitochondriale membraan kodeer. Geen fenotipe is ooit voorheen aan 'n mutant alleel in hierdie geen toegeskryf nie.

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This thesis is dedicated to my wife, Karin.
Hierdie tesis is aan my vrou, Karin, opgedra.

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BIOGRAPHICAL SKETCH

Hentie Swiegers was born in Pretoria, South Africa on the 11th of April 1975. He matriculated with distinction at Menlo Park High School, Pretoria in 1993. Hentie enrolled at the University of Pretoria in 1994 and obtained a B.Sc. *cum laude*, majoring in Biochemistry and Microbiology, in 1996. In 1997 he enrolled at the University of Stellenbosch and obtained a Hons.B.Sc. in Microbiology, the same year. Thereafter, he enrolled for a M.Sc. in Microbiology.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal (Genetics) to which Chapter 3 will be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature review**
"Peroxisome biogenesis: A yeast perspective"

Chapter 3 **Research Results**
"Selection of mutants affected in genes required for carnitine-dependent activities in *Saccharomyces cerevisiae*: Yat1p is an essential component in a carnitine-dependent strain"

Chapter 4 **General Discussion and Conclusions**

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CHAPTER 1

INTRODUCTION AND PROJECT AIMS

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1. INTRODUCTION AND PROJECT AIMS

1.1 THE CLONING OF GENES INVOLVED IN CARNITINE-DEPENDENT ACTIVITIES IN *SACCHAROMYCES CEREVISIAE*

L-Carnitine is a highly polar compound playing a important role in eukaryotic cells. In mammalian cells, many functions have been established where carnitine plays a role, which include the β-oxidation of long-chain fatty acids, the elimination of selective acyl residues and the modulation of CoSH/Acyl-CoA ratio. In addition, carnitine acts as a reservoir of activated acetyl units (Bieber 1988). L-Carnitine has been shown to have positive therapeutic effects in patients with diseases like AIDS, diabetes and Alzheimers (De Simone *et al.* 1993; Carta *et al.* 1999; Keller *et al.* 1998). The reasons for some of these therapeutic effects are unclear. L-Carnitine in high doses is also administered to patients for the treatment of primary carnitine deficiency, a disease in humans caused by metabolic and genetic defects and characterized by low levels of L-carnitine in the serum and/or tissue (Pons and De Vivo 1995).

In contrast to mammalian cells, the only function of carnitine in *S. cerevisiae* that has been described resides in the transfer of activated acyl-groups from the peroxisome to the mitochondria via the carnitine acetylcarnitine shuttle. Activated acetyl groups have to be transferred in the form of an acetylcarnitine since the peroxisomal membrane is impermeable to acetyl-CoA (Van Roermund *et al.* 1995). Acetyl-CoA is formed in the peroxisome of *S. cerevisiae* as a result of the β-oxidation of fatty acids. In yeast, the peroxisome is the sole site for fatty acid oxidation (Kunau *et al.* 1988). Fatty acids are transported to the peroxisome where they are oxidized by the β-oxidation cycle to form two-carbon acyl-groups in the form of acetyl-CoA. These two-carbon acyl-groups are then transferred from the peroxisome to the cell for gluconeogenesis and other anabolic pathways, or used in the tricarboxylic acid cycle (TCA) of the mitochondia to generate ATP. In this way, it is possible for the cell to use fatty acid as a sole carbon source.

The yeast *S. cerevisiae*, is a model organism in the study of various molecular processes that take place in the cell. To date, only four genes have been identified that are implicated in carnitine-dependent activities in this organism: (i) *CAT2*, encoding the carnitine acetyltransferase of the peroxisomal and inner-mitochondrial membrane (Kispal *et al.* 1993); (ii) *YAT1*, encoding the carnitine acetyltransferase of the outer-mitochondrial membrane (Schmalix and Bandlow 1993); (iii) *AGP2*, encoding the plasma membrane carnitine transporter (Van Roermund *et al.* 1999); (iv) *CAC*, the carnitine acylcarnitine translocase of the inner-mitochondrial membrane (Van Roermund *et al.* 1999). Considering the importance of carnitine described in mammalian cells, much work remains

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to be done in *S. cerevisiae* in order to identify the molecular components of carnitine-dependent activities.

1.2 PROJECT AIMS

The specific aims of this study were to:

i) develop a strategy for the cloning of genes involved in carnitine-dependent activities;
ii) clone and identify genes involved in carnitine-dependent activities;
iii) investigate the function of these carnitine-dependent genes;
iv) contribute to the understanding of the role of carnitine in the cell.

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CHAPTER 2

LITERATURE REVIEW

Peroxisome function and biogenesis: A yeast perspective

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2. LITERATURE REVIEW

2.1 INTRODUCTION

In contrast to prokaryotic cells, eukaryotic cells are compartmentalized by membranes. Membrane compartments are called organelles, with each specific organelle possessing a different complement of enzymes that perform unique metabolic reactions. This compartmentalization of cellular functions provides the eukaryotic cell with an additional level of control and creates a favorable environment for the functioning of specific metabolic reactions. It does, however, result in some additional complications, requiring for example protein targeting and mechanisms to overcome membrane barriers. One of the last organelles to be discovered and playing a unique role is the peroxisome.

Peroxisomes are small, single membrane bound organelles approximately 0.2-1 μm in diameter and present in most eukaryotic cells. The name peroxisome was derived from the fact that these organelles produce toxic H_2O_2 (hydrogen peroxide) as a by-product of their metabolic activity (de Duve and Bauduin 1966). The peroxisomes perform a large range of metabolic roles in eukaryotic cells, some of which are common to all organisms like β-oxidation of fatty acids, whereas others , like photorespiration in plant leaves and ether-lipid synthesis in mammalian cells, are specific to the peroxisomes of these organisms (reviewed in Van den Bosch *et al.* 1992).

In yeast, the peroxisome is characterized by the presence of several enzymes involved in metabolic pathways that are crucial for the survival of the cell when grown on certain carbon sources, in particular fatty acids. The β-oxidation cycle present in the peroxisomes of yeast results in the production of carbon intermediates and, with the help of the mitochondria, these intermediates are used to generate energy (reviewed in Kunau *et al.* 1988). Another metabolic process common to the peroxisomes of all organisms studied is the formation of hydrogen peroxide (H_2O_2) and the subsequent decomposition of it to water and oxygen catalyzed by the enzyme catalase. The toxic H_2O_2 is produced as a by-product of β-oxidation, explaining why rapid decomposition is essential (de Duve and Bauduin 1966). In some organisms, the peroxisome fulfils metabolic roles exclusive to that particular organism, like photorespiration which occurs only in peroxisomes of plant leaves (reviewed in Van den Bosch *et al.* 1992). Another unique metabolic pathway occurring only in peroxisomes of yeast and germinating fat-bearing seeds, is the glyoxylate cycle. This cycle is a modified tricarboxylic acid cycle (TCA) that converts acetyl-CoA into TCA carbon intermediates (Armstrong 1989). These carbon intermediates can then be transported to the mitochondria for further metabolism.

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The study of peroxisomes in yeast began with the discovery of their presence in *Saccharomyces cerevisiae* (Avers and Federman 1968). For a long period thereafter, little research was conducted on peroxisomes in this organism. At one stage, their presence in *S. cerevisiae* was even questioned due to the localization of the peroxisome specific catalase to the vacuole (Susani *et al.* 1976). The fact that these studies were conducted under conditions where peroxisomes are not induced probably explains these results. Today we know that peroxisomes in *S. cerevisiae* are strongly induced when fatty acids like oleic acid are utilized as sole carbon source (Veenhuis *et al.* 1987). Since then, the peroxisome in *S. cerevisiae* and other yeast has been the focus of numerous studies trying to establish the precise mechanism of biogenesis.

There are several reasons why yeast have been the perfect organism for studying peroxisomes. First, peroxisomes in yeast are the sole site for β-oxidation, the process of catabolizing fatty acids to acyl-groups (reviewed in Kunau *et al.* 1988). This simplifies the study of β-oxidation in yeast and the isolation of mutants. Second, peroxisomes can be induced in different species of yeast when the strains are grown on certain carbon sources. In the case of *S. cerevisiae*, peroxisomes are induced when growing on media containing oleic acid (Veenhuis *et al.* 1987). The study of the induction of peroxisomes gives the opportunity to gain insights in the processes of proliferation and biogenesis. Third, the yeast peroxisome is dispensable under certain growth conditions, making the isolation of *pex* mutants (mutants affected in peroxisome biogenesis) a simple selection process, since peroxisomal mutants can be obtained that will be viable on the appropriate media. In addition, yeast can be cultured as both diploid and haploid cells which makes a genetic analysis very easy. Last, the vast amount of information and molecular techniques available for yeast has contributed to the speed and ease at which the discovery of the role of peroxisomes and the mechanism of their biogenesis has advanced.

In humans, peroxisomes play an essential role in the metabolism of the cell, particularly in lipid metabolism (reviewed in Van den Bosch *et al.* 1992). Metabolic processes that take place in human peroxisomes include the synthesis of plasmalogens, cholesterol and bile acids. Catabolic pathways include the β-oxidation of very-long-chain fatty acids (VLCFs), long chain dicarboxylic acids and certain unsaturated fatty acids (reviewed in Waterham and Gregg 1997). Genetic disorders affecting peroxisomes are the cause of several diseases in humans, the most common of which is X-linked adrenoleukodystrophy (X-ALD), a disease brought to attention of the general public by the film 'Lorenzo's Oil' (Lazarow *et al.* 1994). In patients with X-ALD, the peroxisome is defective for the degradation of very-long-chain fatty acids (VLCFs) and the subsequent accumulation of these fatty acids leads to the destruction of the myelin sheath in nervous tissues. The cells from patients with certain peroxisomal defects have strikingly similar defects to some yeast mutants affected in peroxisome biogenesis. Yeast can be used as a simple model for understanding these peroxisomal defects (Lazarow 1995). Recently numerous genes

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responsible for human peroxisomal disorders have been cloned based on homology with yeast counterparts. An example is the human *PEX10* gene that was cloned based on homology with the *Hansenula polymorpha PEX10* gene. Transformation of human *PEX10* restored peroxisome biogenesis in transformed fibroblasts from Zellweger patients of complementation group B (Okumoto *et al.* 1998).

The potentially devastating effect of a peroxisome biogenesis defect in a cell can be anticipated when the importance of its metabolic functions are considered. It is clear that the peroxisome is a vital organelle needed for the survival of eukaryotic cells in a challenging environment. During the last few years great advances have been made in the elucidation of the components and mechanisms of peroxisome biogenesis. Yeast are now the model organisms of choice for these studies. In the following chapters we will be looking into the various aspects of peroxisome structure, proliferation, segregation and especially protein import in the yeast system. We will also discuss the different functions of the peroxisome in yeast and what part they play in cellular metabolism. The parallels that can be drawn between human peroxisomal biogenesis defects and that of peroxisome biogenesis defects in yeast are especially important.

2.2 STRUCTURE OF PEROXISOMES

Like other organelles in the cell, the peroxisome has particular structural characteristics that can be distinguished. Peroxisomes can be observed in cells of yeast, plants and mammals, and are present within most human cell types except in the mature erythrocyte. The number of peroxisomes varies considerably depending on the tissue and cell type. In yeast, peroxisomes are induced when grown on certain carbon sources and the number of peroxisomes in the cell can be seen to increase significantly (Veenhuis and Harder 1991).

In human fibroblast, peroxisomes are observed as circular structures, bounded by a single membrane. Inside the peroxisome, a fine granular matrix can be observed that contains the peroxisomal matrix enzymes (Lazarow 1995). Peroxisomes can fundamentally be described as 'enzyme bags'. One structural characteristic of peroxisomes is the unusually high matrix protein content. This might be due to the fact that most of the enzymes involved in the different metabolic pathways inside the peroxisome are located in the matrix and not bound to the membranes. In contrast to this, the overall protein content of the peroxisomal membrane is relatively low. This can be observed in the typically smooth fracture faces of peroxisomes in freeze-etch replicas indicating the low abundance of membrane proteins (**Fig. 2.1**) (reviewed in Van der Klei and Veenhuis 1997).

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Fig. 2.1. Freeze-etch replica of *Hansenula polymorpha* grown on methanol. The typical smooth fracture faces is observable for the peroxisome (P) indicating low abundance of membrane proteins. Other organelles observed is a mitochondrion (M), endoplasmic reticulum (ER) and a vacuole (V) (taken from Van der Klei and Veenhuis 1997).

Generally, peroxisomes are observed as circular profiles in electron microscopic thin profiles. In *S. cerevisiae* at least one peroxisome is found per cell when grown on glucose. These peroxisomes are small, ranging between 0.1 and 0.2 μm, and are mostly found near the plasma membrane (**Fig. 2.2A**). Peroxisomes of cells grown on glycerol or ethanol are somewhat bigger (**Fig. 2.2B**). Those grown on oleic acid are significantly bigger and also more abundant (**Fig. 2.2C**) (Lazarow and Kunau 1996).

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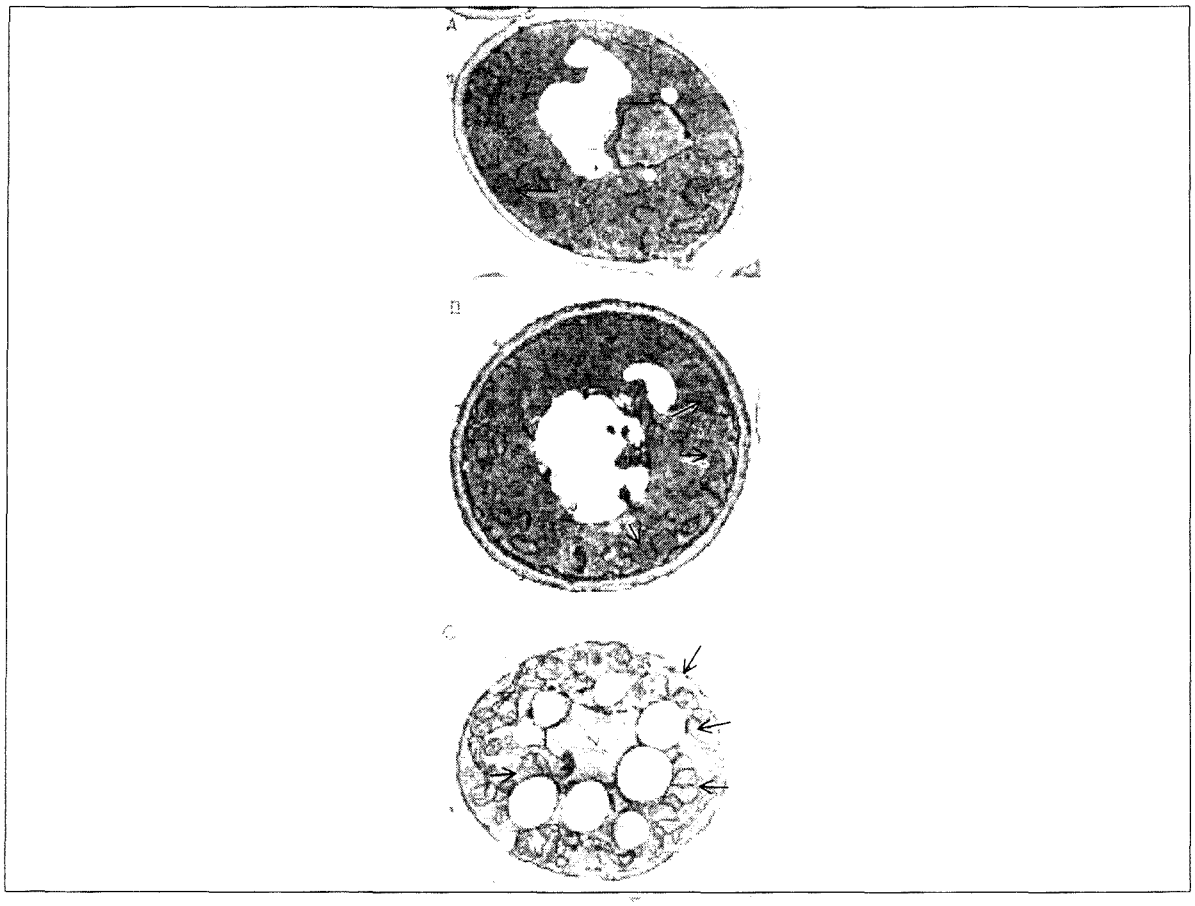

Fig.2.2. Electron micrograph of peroxisomes of *S. cerevisiae* cells grown on glucose (A), ethanol (B) and oleic acid (C) (taken from Lazarow and Kunau 1997).

While metabolic activities which in normal cells occur in the peroxisome, can take place in mutants devoid of peroxisomes, this defect leads to a severe energetic and metabolic disadvantage causing the cells to grow slowly (Van der Klei *et al.* 1991). In this case, the peroxisomal enzymes involved in these metabolic activities are normally transcribed and active, but they are present in the cytosol. The absence of intact peroxisomes also results in metabolism of H_2O_2 , mediated no longer by peroxisomal catalase, but by catalase in the cytosol (Van der Klei *et al.* 1991).

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2.3 METABOLIC FUNCTIONS OF PEROXISOMES

2.3.1 Introduction

Peroxisomes are organelles housing a variety of enzymes playing parts in various essential metabolic pathways. In yeast, the peroxisome is mainly involved in the metabolism of non-fermentable carbon sources like fatty acids and, in some yeast, methanol (Veenhuis *et al.* 1987; Van der Klei *et al.* 1991). The peroxisome is the compartment where the catabolism of fatty acids takes place, and for this purpose a distinct biochemical pathway called β-oxidation is needed (reviewed in Kunau *et al.* 1988). The peroxisome also contains enzymes involved in the metabolism of methanol in methylotrophic yeast like *H. polymorpha* (Van der Klei *et al.* 1991). Along with β-oxidation, two other characteristic metabolic functions exist in peroxisomes of yeast: peroxisomal oxidation and the glyoxylate cycle. Peroxisomal oxidation is the first function of peroxisomes discovered and is based upon the formation of hydrogen peroxide and its decomposition by catalase (de Duve and Bauduin 1966). The glyoxylate cycle is a modified TCA cycle, in most cases completely contained in the peroxisome, and is unique to yeast and cells of germinating fat-bearing seed (reviewed in Van den Bosch *et al.* 1992). In the following sections, these pathways will be discussed in more detail.

2.3.2 Peroxisomal respiration

Peroxisomal respiration refers to the formation and decomposition of hydrogen peroxide and was the first function identified in peroxisomes (de Duve and Bauduin 1966). Hydrogen peroxide is toxic to the cell and is decomposed by the enzyme catalase A, encoded by the gene *CTA1* in *S. cerevisiae* (Cohen *et al.* 1988). Additional cellular protection against H_2O_2 is provided in the cytosol of *S. cerevisiae* by catalase T, encoded by *CTT1* (Hartig and Ruis 1986). Hydrogen peroxide is produced as a by-product in the first step of β-oxidation through the regeneration of FAD (**Fig. 2.3**). Peroxisomal respiration does not conserve energy through the formation of ATP, but loses it in the form of heat (de Duve and Bauduin 1966). The H_2O_2 serves no purpose and has to be quickly disposed of.

2.3.3 Peroxisomal β-oxidation of fatty acids

In yeast, the β-oxidation system is fully contained in the peroxisome, not as in the case of mammalian cells where it takes place partially in the peroxisome and partially in the mitochondrium (reviewed in Kunau *et al.* 1988). The β-oxidation cycle is essential for the catabolism of fatty acids in all organisms. In yeast, a mutation in one of the genes required

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for β-oxidation would render cells not viable on fatty acid media. By selecting such yeast mutants, it is possible to clone genes involved in β-oxidation (Dmochowska *et al.* 1990; Hiltunen *et al.* 1992). The fact that the β-oxidation cycle is confined to the peroxisome makes isolation of these mutants less complicated. The β-oxidation cycle is illustrated in **Fig. 2.3**.

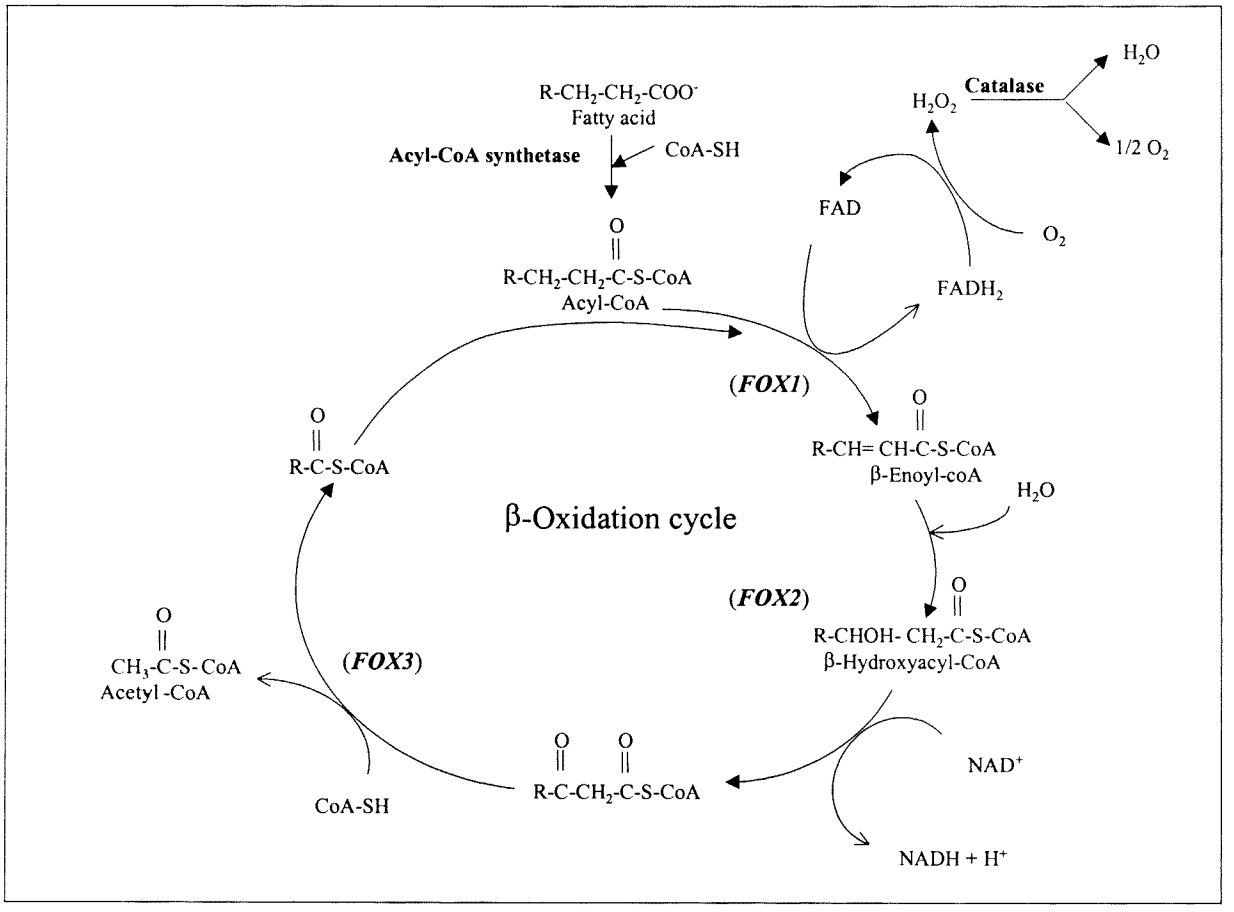

Fig. 2.3. The β-oxidation cyle (adapted from Armstrong 1989).

In order for a fatty acid to be oxidized it first has to be activated. This takes place with the help of an acyl-CoA-synthetase that covalently links a CoA-SH molecule to the fatty acid. After activation the fatty acid is ready to by oxidized in the β-oxidation cycle (reviewed in Kunau *et al.* 1988). The first enzyme in the β-oxidation cycle is the acetyl-CoA oxidase, encoded by the *FOX1/POX1* gene in *S. cerevisiae* (Dmochowska *et al.* 1990). This enzyme converts the activated fatty acid that is transported into the peroxisome into the corresponding α,β-trans-unsaturated enoyl-CoA. The next two reactions in the cycle are catalyzed by a multi-functional protein encoded by the *FOX2* gene, converting this unsaturated enoyl-CoA ester to the corresponding β-oxoacyl-CoA ester. The *FOX2* gene was cloned in *S. cerevisiae* by functional complementation of the *fox2* mutant (Hiltunen *et al.* 1992). The last step uses the enzyme thiolase, encoded by *FOX3/POT1* in

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S. cerevisiae, to cleave this product into an acetyl-CoA group and an activated fatty acid group ready to complete another cycle (reviewed in Kunau *et al.* 1988). The cycle continues, each time generating one acetyl-CoA until the complete fatty acid is oxidized. The activated acyl group can then either be shuttled to the mitochondria by the carnitine acetylcarnitine shuttle for further metabolism via the TCA cycle or be incorporated into the glyoxylate cycle where it is used as a building block for the synthesis of TCA intermediates like succinate (Van Roermund *et al.* 1996).

2.3.4 Glyoxylate cycle

In contrast to mammals, germinating fat-bearing seeds and yeast are able to use fatty acids as a sole carbon source (reviewed in Van den Bosch *et al.* 1992). This ability is partly due to the existence of the glyoxylate cycle present in the peroxisome. The glyoxylate cycle is a modified TCA cycle that is able to use the catabolic end-product of fatty acid oxidation, acetyl-CoA, to synthesize TCA intermediates like succinate for anabolic pathways (Armstrong 1989). The glyoxylate cycle is illustrated in **Fig. 2.4**. The acetyl-CoA condenses with oxaloacetate to produce citrate in a reaction catalyzed by citrate synthetase (CS). Peroxisomal citrate synthetase in *S. cerevisiae* is encoded by the *CIT2* gene (Lewin *et al* 1990). The citrate synthetase in the peroxisomal glyoxylate cycle is the isozyme of the citrate synthetase encoded by *CIT1* of the mitochondrial TCA cycle (Rosenkrantz *et al* 1986). In the next step the citrate is converted into isocitrate. Then, in a reaction unique to the glyoxylate cycle, isocitrate is cleaved into succinate and glyoxylate by isocitrate lyase (ICL). Following this, another reaction unique to this cycle results in the condensation of a second acetyl-CoA with glyoxylate to form malate in a reaction catalyzed by malate synthetase (MS). The cycle is completed by the oxidation of malate to oxaloacetate by the enzyme malate dehydrogenase (MDH) (Armstrong 1989). The result of this cycle is therefore the net production of the C_4 compound succinate from two molecules of acetyl-CoA. This succinate can be used for anabolic purposes or for the production of energy as a TCA cycle intermediate.

The peroxisomes of *S. cerevisiae* contain two isozymes of MDH. One is the product of *MDH2*. The Mdh2p does not have a peroxisomal targeting signal but some of the enzyme was found in the peroxisomes of oleate grown cells. *MDH2* is essential for growth on acetate (McCammon *et al.* 1990). The second isozyme is encoded by *MDH3* (Steffan and McAlister-Henn 1992). The Mdh3p is essential for growth on oleate and is responsible for the reoxidation of NADH formed within peroxisomes during β-oxidation (Van Roermund *et al.* 1995). The other MDH isozyme present in *S. cerevisiae* is Mdh1p which is active in the TCA cycle of the mitochondria (Thompson *et al.* 1988).

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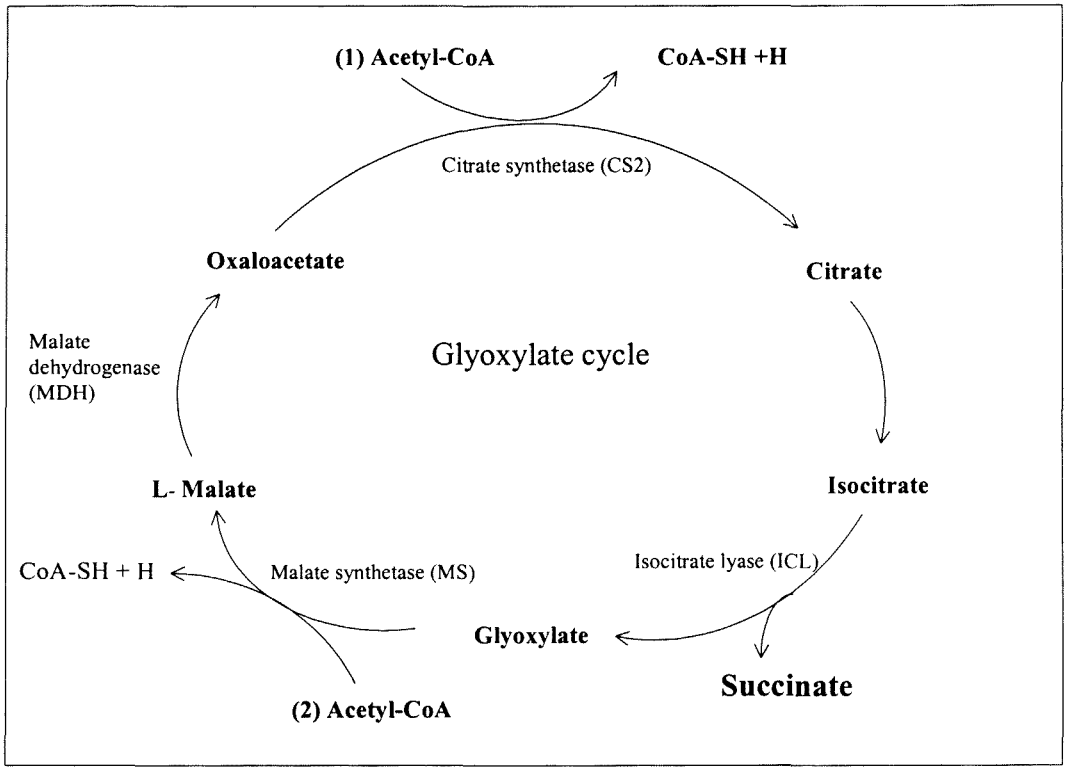

Fig. 2.4. The glyoxylate cycle (adapted from Armstrong 1989).

The peroxisomal localization of all of the enzymes of the glyoxylate cycle has been established for all yeast studied except for *S. cerevisiae*. In these yeast, the enzymes of the glyoxylate cycle isocitrate lyase (ICL), malate synthetase (MS), malate dehydrogenase (MDH) and citrate synthetase (CS2) are all located in the peroxisomes (reviewed in Van der Klei *et al.* 1997). In *S. cerevisiae,* the location of the entire glyoxylate cycle to the peroxisome has not been proven unequivocally. In this yeast, activities of malate synthase (McCammon *et al* 1990), citrate synthase (Lewin *et al* 1990) and malate dehydrogenase (Minard *et al* 1991) were demonstrated to be peroxisomal. However, the enzyme isocitrate lyase (IL) was found to be active only in the cytosol (Taylor *et al* 1996). This shows that the presence of isocitrate lyase in peroxisomes is not essential for a functional glyoxylate cycle in *S. cerevisiae*. This also means that glyoxylate cycle intermediates citrate and malate have to cycle between the peroxisomes and the cytosol. The reason for these additional transport steps is unknown.

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2.3.5 Other functions associated with peroxisomes

A function unique to the peroxisomes of methylotrophic yeast like *H. polymorpha* and *Pichia pastoris*, is the metabolism of methanol (Sibirny *et al.* 1988; Van der Klei *et al.* 1991). Methanol is metabolized within the peroxisome with the help of the enzymes alcohol oxidase (AOX) to form formaldehyde. This compound is converted to formate by the enzyme formaldehyde dehydrogenase. Formate is converted to carbon dioxide by the enzyme formate dehydrogenase. As a by-product of this process, hydrogen peroxide is formed which is subsequently decomposed by catalase (Sibirny *et al.* 1988; Van der Klei *et al.* 1991).

There are some other less well described metabolic functions that occur in the peroxisomes of yeast. These functions are related to nitrogen metabolism, spore formation and transamination. The glyoxylate cycle is indeed involved in some aspects of nitrogen metabolism. Glyoxylate is a by-product formed when allantoin is catabolyzed as a nitrogen source. Accumulation of glyoxylate is toxic to the cell, and the substance has to be removed by condensing with acetyl-CoA to form malate. This reaction is catalyzed by an isozyme of malate synthetase encoded by *DAL7* in *S. cerevisiae* (Hartig *et al.* 1992). The Dal7p has a peroxisomal targeting signal and is likely to be peroxisomal, but this has not yet been proven experimentally.

The *SPS19* gene encodes a protein involved in sporulation in *S. cerevisiae* and has a peroxisomal targeting signal (Coe *et al.* 1994). Deletion of *SPS19* in conjunction with the deletion of another sporulation-specific gene, *SPS18,* results in very low sporulation efficiency. The spores are also much less resistant to ether, suggesting a possible role of the peroxisomal enzyme in spore-wall formation (Coe *et al.* 1994).

Transamination is a reaction common to peroxisomes in various organisms. The gene *AAT2* in *S. cerevisiae* encodes an aspartate aminotransferase with a peroxisomal targeting signal. When *S. cerevisiae* is grown on oleic acid, the Aatp is localized in the peroxisome, whereas it is localized in the cytosol when grown on glucose. However, the *AAT2* gene is non-essential for growth on oleic acid (Verleur *et al.* 1997).

Further work needs to be done to understand these and other functions in the peroxisome. However, it is clear from the functions discussed in this section that the peroxisome contribute to some vital metabolic processes in yeast, highlighting the importance of the organelle in this organism.

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2.4 TRANSPORT OF METABOLITES

Peroxisomal membranes are impermeable to most metabolites and for this reason specific transport mechanisms are required for exchange of metabolites with the cytoplasm. Several stages where transport is essential have been distinguished. Some important steps include the transport of fatty acids across the peroxisomal membrane (Hettema *et al.* 1996). Also, acetyl-CoA and NADH, the products of fatty acid β-oxidation have to be transported out of the peroxisome for further metabolism in other parts of the cell (Van Roermund *et al.* 1995).

Transport of fatty acids into the peroxisome of *S. cerevisiae* is divided into two processes. Medium-chain fatty acids (MCFAs) probably enter through diffusion or with the help of an as yet unknown transporter. Once these fatty acids are inside the peroxisome, they are activated for β-oxidation by the peroxisomal fatty acid activator of *S. cerevisiae*, Faa2p (Hettema *et al.* 1996). Long-chain fatty acids (LCFAs) are, however, first activated in the cytosol before being transported via the peroxisomal ABC transporter-complex Pat1p and Pxa1p (Hettema *et al.* 1996).). It is unclear if these proteins transport the acid-CoA ester or if the CoA has been replaced by a carnitine group, as is the case for higher eukaryotes which contain carnitine octanoyltransferases and carnitine palmitoyltransferases (reviewed in Bieber 1988). However, in *S. cerevisiae* activity for these transferases could not be detected (Schmalix and Bandlow 1993). X-linked adrenoleukodystrophy (X-ALD), the disease mentioned earlier (p.5), is characterized by high levels of VLCFAs in the serum which is caused by a decreased rate of VLCFA β-oxidation (Wanders *et al.* 1992). The gene responsible for this disease was cloned and identified as coding for an ATP binding cassette (ABC) protein (Mosser *et al.* 1994). It might be that in these patients this protein is encoded by a mutated gene responsible for a VLCFAs transport deficiency, resulting in high levels of VLCFAs (Valle and Gärtner 1993).

The products of β-oxidation, NADH and acetyl-CoA, can not diffuse across the peroxisomal membrane of *S. cerevisiae* (Van Roermund *et al.* 1995). One way of transporting products across impermeable membranes is through shuttle-systems like those common to mitochondria (Walker and Runswick 1993). Instead of transporting NADH itself, electrons are transferred from NADH to certain metabolites acting as reducing equivalents as in the case of the malate/ aspartate shuttle of the mitochondria (Armstrong 1989). Through this process NADH can be oxidized to NAD^+ without leaving the peroxisome. In *S. cerevisiae* it is most probable that an oxaloacetate/ malate and malate/ aspartate shuttle exist, allowing the regeneration of NADH+ by the peroxisomal malate dehydrogenase (*MDH3*), which reduces oxaloacetate, followed by a shuttling of malate across the peroxisomal membrane (Van Roermund *et al.* 1995).

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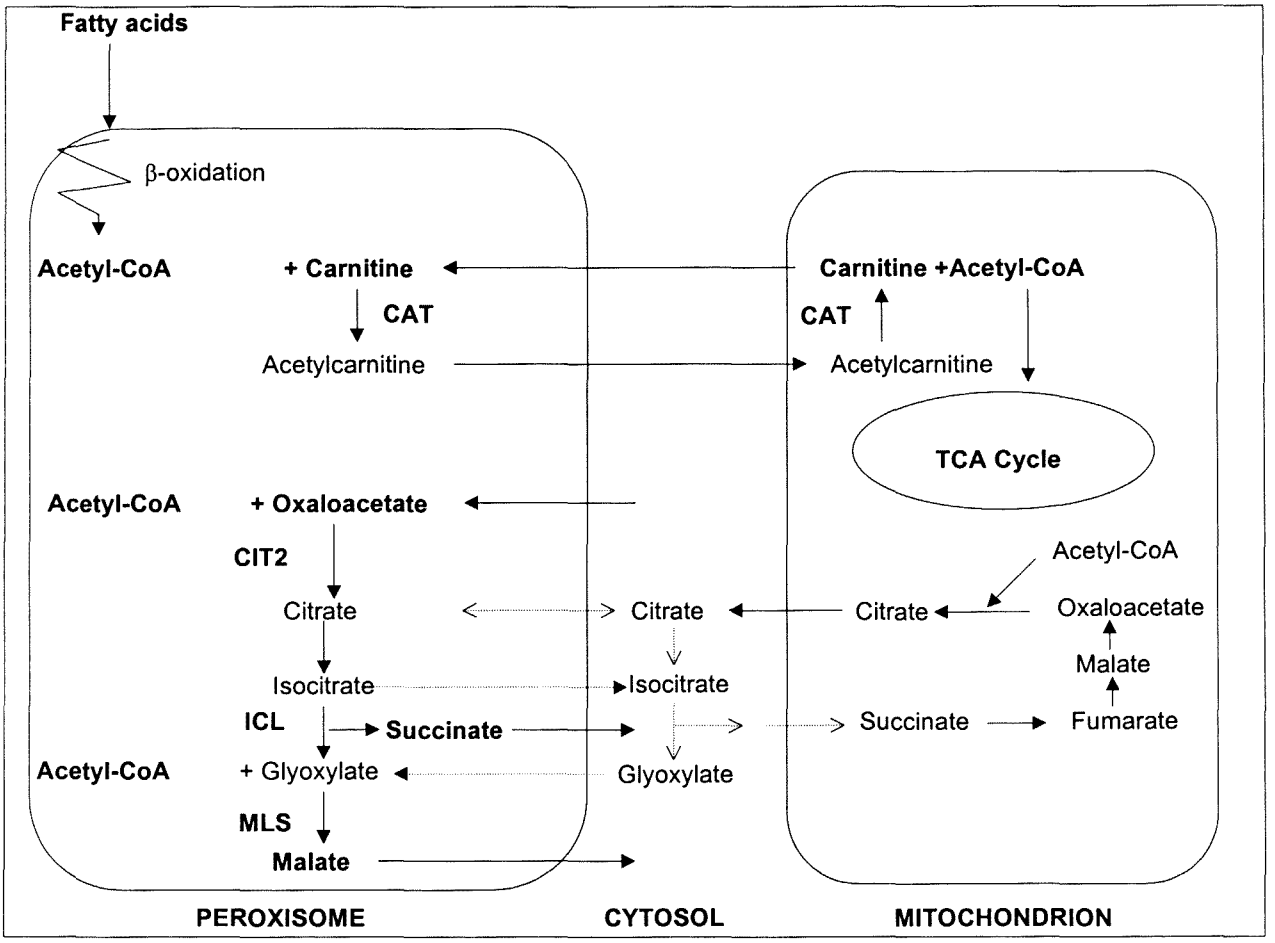

Fig. 2.5 Metabolic model for the transport of activated acyl-groups from the peroxisome to the mitochondrion in *S. cerevisiae*. The abbreviations are peroxisomal citrate synthetase (CIT2), isocitrate lyase (ICL), malate synthetase (MLS) and carnitine acetyltransferase (CAT) (adapted from Van Roermund *et al.* 1995).

Acetyl-CoA can also not be transported out of the peroxisome, but the activated acyl group can be transported when bound to carnitine through the carnitine acetylcarnitine shuttle. The acetyl group of acetyl-CoA is transferred to carnitine to form an acetylcarnitine ester with the help of a carnitine acetyltransferase (CAT). The acetylcarnitine ester can be transported out of the peroxisome where acetyl-CoA can be re-formed by releasing the carnitine (reviewed in Bieber 1988). The transport of activated acyl-groups is illustrated in **Fig. 2.5**. Two carnitine acetyltransferases have been cloned in *S. cerevisiae*. The major carnitine acetyltransferase encoded by *CAT2* contributes to >95% of the total CAT activity (Kispal *et al.* 1993). The second carnitine acetyltransferase, encoded by the *YAT1* gene, is located on the outer mitochondrial membrane and is probably responsible for the remaining 5% or so of the total CAT activity (Schmalix and Bandlow 1993). The carnitine acetyltransferse encoded by *CAT2* is differentially targeted to the peroxisomes and the inner mitochondrial membrane. The *CAT2* encoded protein contains mitochondrial

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targeting signals as well as peroxisomal targeting signals and localization is controlled at transcriptional or translational level (Elgersma *et al.* 1995). An alternative way of removing acetyl-CoA from the peroxisome is via the glyoxylate cycle. The acetyl-CoA enters the glyoxylate cycle resulting in the formation of succinate, which can then be transported out of the peroxisome (Van Roermund *et al.* 1995). The transport of succinate probably occurs via the putative dicarboxylate carrier, Acr1p (Palmieri *et al.* 1997). These observations are supported by the fact that disruption of either *CIT2*, the citrate synthetase of the glyoxylate cycle, or *CAT2* alone does not result in growth defects on oleic acid media. However, if both *CIT2* and *CAT2* are disrupted, the cells are no longer able to grow on oleic acid media, strongly suggesting that acetyl groups can only be removed from the peroxisome via either the glyoxylate cycle or the carnitine acetylcarnitine shuttle (Van Roermund *et al.* 1995). There are therefore only two pathways by which activated acetyl groups can be removed from the peroxisome.

From the information discussed above, it is clear that the peroxisome is responsible for some vital metabolic processes in eukaryotic cells. We have also seen the benefits provided by the closed compartment and the additional complications it causes, for example the necessity for the transport of metabolites through the peroxisomal membrane. In yeast, the importance of this organelle in the metabolism of some non-fermentable carbon sources like fatty acids is clear. Peroxisomes are not redundant when grown on these carbon sources. Also, the organelle is crucial in the decomposition of the highly toxic substance hydrogen peroxide. Peroxisomes confine these reactions in a closed compartment and can therefore be described as 'enzyme bags'. The next sections will concentrate on the actual formation or biogenesis of these interesting organelles.

2.5 PEROXISOME BIOGENESIS IN YEAST

2.5.1 Introduction

To understand the function of peroxisomes, it is important to know how these organelles are formed. Most progress achieved thus far concerning this aspect is due to the genetic analysis of yeast mutants. The use of different yeast species in the study of peroxisome biogenesis has proven to be of great importance. Due to the vast amount of genetic and biochemical information available for yeast, and the possibility to control the biogenesis of their organelles through growth on certain substrates, these organisms have become very successful model systems for the dissection of the mechanisms of peroxisome biogenesis (Veenhuis and Harder 1991). The isolation of yeast peroxisome biogenesis mutants has yielded many of the genes involved in this process. The yeast mutants also serve as

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excellent models for similar defects that occur in humans. Peroxisomal disorder genes that have been cloned through the knowledge of a yeast gene include the human PTS1 (peroxisome targeting signal receptor 1) receptor (Wiemer *et al*, 1995; Dodt *et al*, 1995). This is the gene responsible for Zellweger syndrome and, in some patients, adrenoleukodystrophy. The gene was discovered due to the similar phenotypes of yeast mutants *P. pastoris* and *S. cerevisiae pex5* and cells from patients belonging to complementation group 2. Both human and yeast mutants had similar import-deficiencies (Wiemer *et al*, 1995; Dodt *et al*, 1995). It is clear that the yeast model has played a very important role in identification of disease genes relating to the peroxisome. Much of the functioning of the peroxisome in humans can be understood through knowledge gained by studying peroxisome biogenesis in yeast.

2.5.2 The isolation of peroxisome biogenesis mutants

Mutants defective in peroxisome biogenesis have been isolated in various yeast species, including *S. cerevisiae*, *P. pastoris*, *H. polymorpha* and *Yarrowia lipolytica*. In the past, these mutants where given such diverse names as *pas*, *per*, *pay* or *peb*, but are now named *pex* in an effort to unify yeast peroxisome biogenesis gene and protein nomenclature (Distel *et al*. 1996). Proteins involved in peroxisome biogenesis (inclusive of peroxisomal matrix protein import, membrane biogenesis, peroxisome proliferation and peroxisome inheritance) are designated peroxins, with *PEX* representing the gene acronym. Proteins involved in peroxisomal metabolic processes (eg. *FOX1* in β-oxidation) or transcription factors that may affect peroxisome proliferation and/or morphology when mutated, are not included in this group.

In *S. cerevisiae*, the peroxisome is essential when growing on the fatty acid oleate. Peroxisome proliferation is also induced on fatty acid oleate media in this yeast (Veenhuis *et al*. 1987). Mutants with defects in peroxisomes could easily be identified through screening of genes defective for growth on these carbon sources (Erdmann *et al*. 1989). These oleate non-utilizing mutants (*onu*), which are either fatty acid oxidation mutants or peroxisome assembly mutants (*pex*), were analyzed. Electron microscopy, subcellular fractionation of organelles and genetic analysis of these mutants resulted in the isolation of 12 *pex* mutant complementation groups (Erdmann 1992). This is a negative selection procedure and is very laborious. To overcome this problem, a positive selection procedure based on the lethality of hydrogen peroxide was developed in *S. cerevisiae* (Van der Leij *et al*. 1992). Hydrogen peroxide is produced in yeast during β-oxidation of fatty acids, and upon addition of the catalase inhibitor 3-AT accumulates, causing the cell to die. Cells that are in any way affected in the peroxisome will not produce hydrogen peroxide, and subsequently the cells will survive. Despite this enrichment procedure, only 2% of the surviving cells were oleate non-utilizing mutants. Another disadvantage of this method is

that the selection is not selective for peroxisome assembly but also resulted in the isolation of fatty acid oxidation mutants. A selection procedure using bleomycin import circumvented this problem (Elgersma *et al.* 1993). This selection method is illustrated in **Fig. 2.6**.

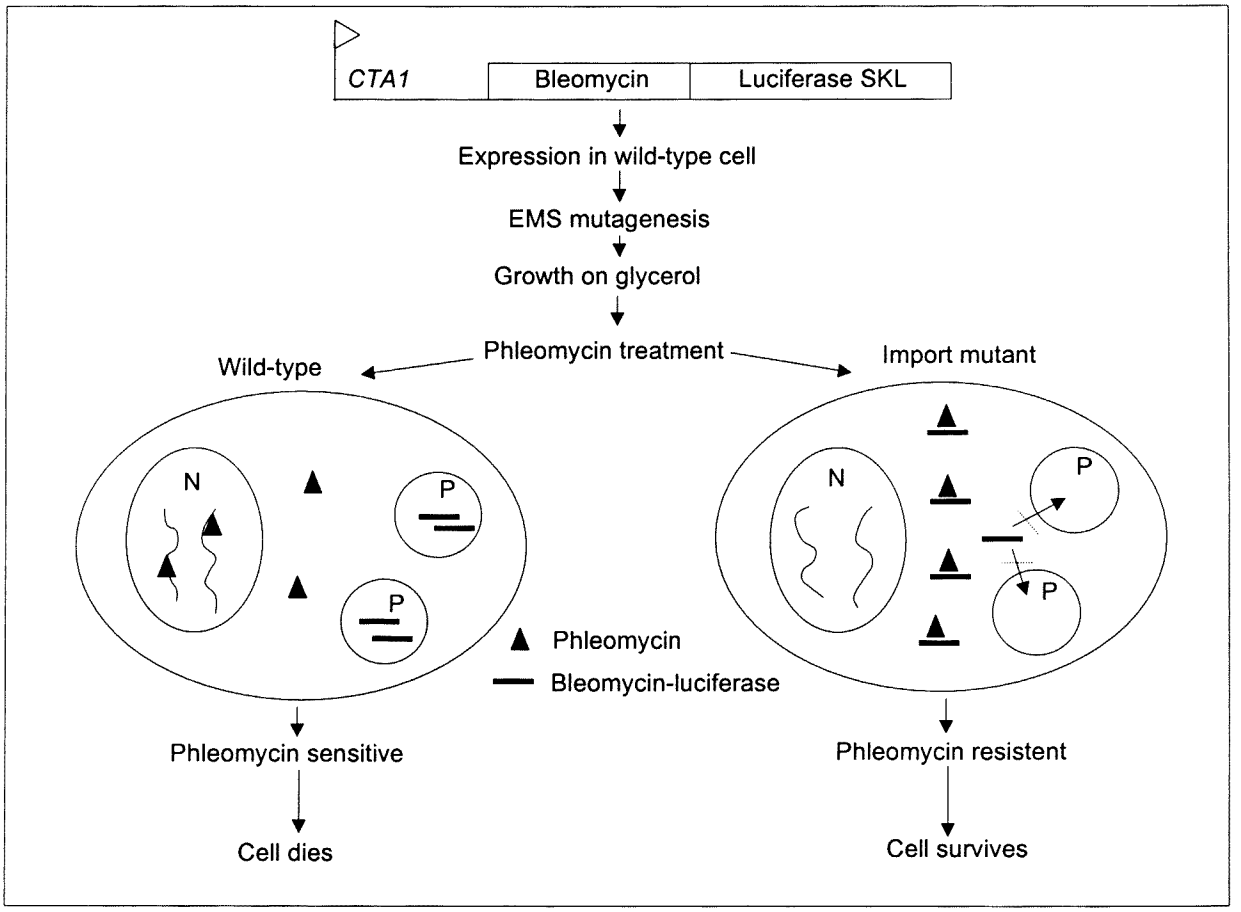

Fig. 2.6. Selection scheme for the isolation of peroxisomal import and/or peroxisome assembly mutants using the bleomycin luciferase protein. *S. cerevisiae* cells transformed with the chimeric bleomycin-luciferase gene were mutagenized. These cells were treated with phleomycin and plated. Wild-type cells are sensitive to the toxic phleomycin because the bleomycin protein is imported into the peroxisome, thereby preventing an interaction of the bleomycin protein with the phleomycin ligand. A mutation preventing the import of the bleomycin protein unites this protein with the phleomycin ligand and results in an increased resistance to phleomycin. P=peroxisome, N=nucleus (adapted from Elgersma *et al.* 1993).

In this procedure a chimeric gene was constructed encoding the bleomycin resistance protein linked to the peroxisomal protein luciferase. In the presence of the toxic pleomycin ligand, wild type cell take up the neutralizing action of the chimeric protein into the peroxisome, and subsequently, the cells die. Peroxisomal import and assembly mutants

are unable to take up the chimeric protein, which will subsequently reside in the cytosol, making the cell resistant to the toxic effect by binding the pleomycin ligand. The selection procedure is very efficient, and upon mutagenesis, the amount of oleic acid non-utilizing mutants (*onu*) was 10% of the total resistant colonies. The procedure is also very specific for import and assembly mutants since all of the *onu* mutants were peroxisomal import/ assembly mutants (Elgersma *et al.* 1993).

The methylotrophic yeast *P. pastoris* grows well on both methanol and oleate. This phenotype was used to identify peroxisomal mutants by selecting for methanol and oleate non-utilizing mutants (*onu*, *mut*) and subsequent analysis by means of electron microscopy and subcellular fractionation of organelles (Lui *et al.* 1992). Recently two novel schemes for the direct selection of *pex* mutants in *P. pastoris* were developed (Johnson *et al.* 1999). These selection schemes are illustrated in **Fig. 2.7**.

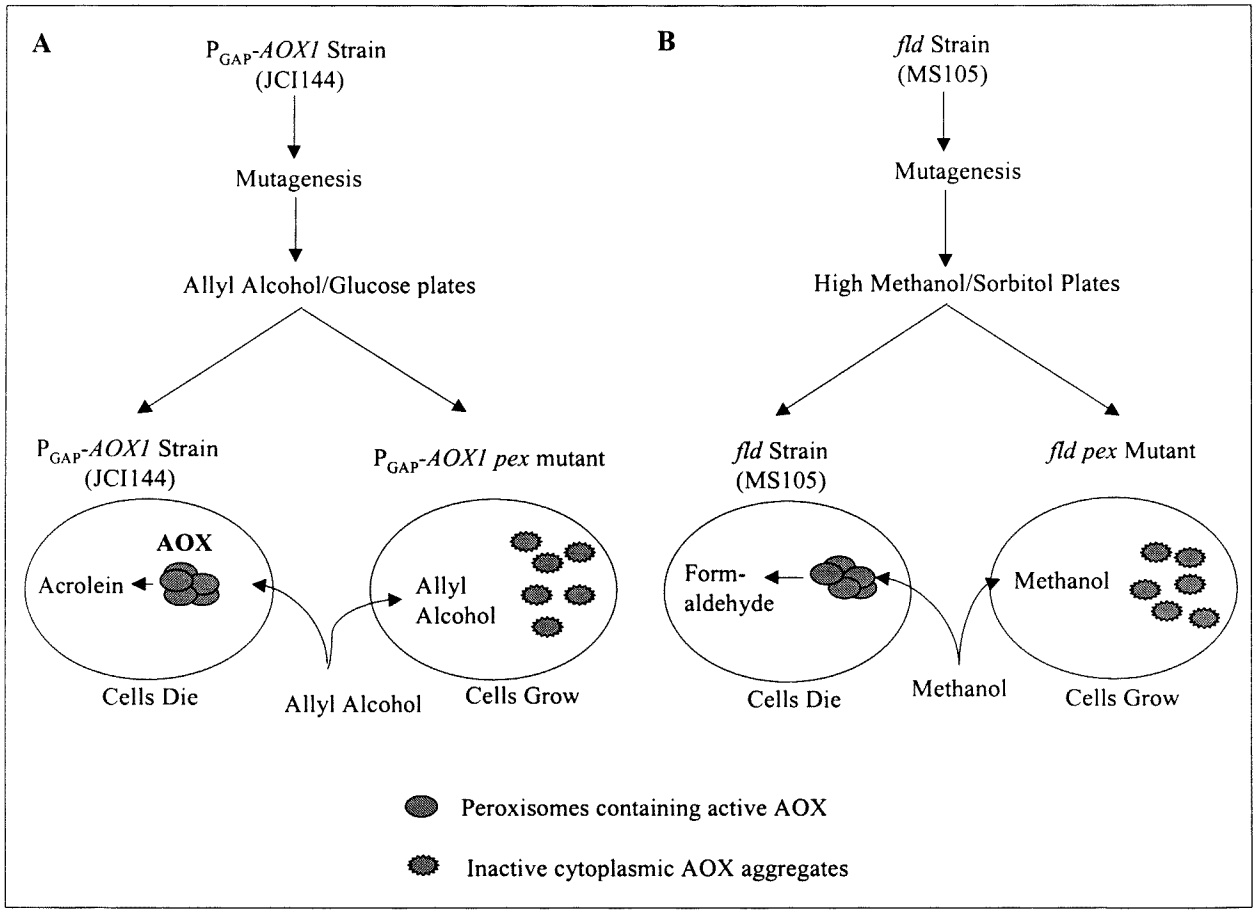

Fig. 2.7 (A) Allyl alcohol selection scheme for the isolation of *P. pastoris pex* mutants. *AOX1* is constitutively expressed from P_{GAP}. Wild-type cells die as a result of active AOX converting allyl alcohol to acrolein. The *pex* mutants grow due to the absence of AOX. (B) Methanol selection scheme for the isolation of *P. pastoris pex* mutants using a *fld1* mutant strain. The *fld1* non-*pex* cells die due to the accumulation of formaldehyde while *fld1 pex* mutants that lack AOX accumulate no formaldehyde and grow (Johnson *et al.* 1999).

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Both schemes take advantage of the fact that methanol-induced *pex* mutants contain little or no alcohol oxidase (AOX) activity due to the enzyme not being assembled properly, causing it to aggregate in the cytosol where it is inactive (Lui *et al.* 1992). The AOX is a peroxisomal matrix enzyme that catalyzes the first step in the methanol-utilisation pathway. The first scheme utilizes allyl alcohol, a compound that is not toxic to cells but is oxidized by AOX to acrolein, a compound that is toxic (Sibirny *et al.* 1988). A problem to be overcome is that *pex* mutants do not grow on methanol. A strain had to be constructed that expressed AOX on glucose media. This strain, JC144, was made by transforming *Pichia pastoris* with a vector that expresses AOX under a constitutive promotor. Exposure of this strain to mutagenesis leads to populations of AOX-induced cells that convert allyl alcohol to acrolein and selectively kills these AOX-containing cells. However, *pex* mutants without active AOX are able to grow and thus positively selected (**Fig. 2.7A**). The second scheme utilizes a *P. pastoris* strain that is defective in formaldehyde dehydrogenase (FLD), a methanol pathway enzyme required to metabolize formaldehyde, the product of AOX. The AOX-induced cells of fld1 strains are sensitive to methanol because of the accumulation of formaldehyde. However, *fld1 pex* mutants, with little active AOX, do not efficiently oxidize methanol to formaldehyde and therefore are not sensitive to methanol (**Fig. 2.7B**). Using these two selection schemes, new *pex* mutant alleles in previously identified *PEX* genes have been isolated along with mutants in three previously unidentified *PEX* groups (Johnson *et al.* 1999).

Through the separate mutant selection strategies used in different species of yeast, several factors of the peroxisomes biogenesis machinery have been identified. The different yeast species have proven to be a perfect model system for analyzing the factors involved in peroxisome biogenesis. The mutagenesis schemes used are specific and the selection process relatively easy. This explains why most of the peroxins identified up to date are yeast peroxins. Up to now, 22 *PEX* genes have been identified using peroxisome biogenesis mutants (Table 1). Novel screening methods will in future allow for the identification of even more components.

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Table 1. List of *PEX* genes and the peroxin characteristics. *Sc- S. cerevisiae*; *Pp- P. Pastoris*; *Hp- H. polymorpha*; *Yl- Y. lipolytica*.

***PEX* Gene**	**Peroxin Characteristics**	**Formerly**
PEX1	117-127 kDa AAA ATPase; subcellular distributiion is unknown.	*ScPAS1; PpPAS1*;
PEX2	C3HC4 zinc-binding integral peroxisomal membrane protein; 35-52 kDa.	*PpPER6; ScPAS5*
PEX3	51-52 kDa integral peroxisomal membrane protein lacking similarity to other proteins.	*ScPAS3; HpPER9*
PEX4	ubiquitin-conjugating enzyme.	*ScPAS2; PpPAS4*
PEX5	PTS1 receptor; 64-69 kDa protein containing 8-9 tetratricopeptide repeats; localized to the cytoplasm and peroxisome.	*PpPAS8; ScPAS10; HpPER3; HpPAH2; YlPAY32*
PEX6	Belongs to the AAA family of ATPases; 112-127kDa; localized to cytoplasm and peroxisome.	*PpPAS5; ScPAS8; YlPAY4 (25)*
PEX7	PTS2 receptor; 42 kDa protein containing six WD40 repeats; localized to the cytosol and peroxisome.	*ScPAS7; ScPEB1;*
PEX8	71-81 kDa peroxisome-associated protein containing a PTS1 signal.	*HpPER1; PpPER3; ScPAS6*
PEX9	42 kDa integral peroxisomal membrane protein lacking similarity to other proteins.	*YlPAY2*
PEX10	C3HC4 zinc-binding integral peroxisomal membrane protein; 34-48 kDa.	*HpPER8; PpPAS7; ScPAS4*
PEX11	27-32 kDa peroxisome-associated protein involved in peroxisome proliferation.	*ScPMP27*
PEX12	48 kDa C3HC4 zinc-binding integral peroxisomal membrane protein.	*PpPAS10; ScPAS11*
PEX13	SH3-containing, 40-43 kDa integral peroxisomal membrane protein; binds the PTS1 receptor.	*ScPAS20; PpPAS6*
PEX14	38 kDa peroxisome associated protein, binds both PTS1 and PTS2 receptor and Pex13p-SH3.	*HpPEX14; ScPEX14*
PEX15	44 kDa phosphorylated integral peroxisomal membrane protein.	*ScPAS21*
PEX16	44 kDa peripheral protein located at the matrix face of the peroxisomal membrane.	*YlPEX16*
PEX17	23 kDa peroxisome associated protein, binds Pex14p.	*ScPAS9*
PEX18	Essential for targeting of proteins via PTS2.	*ScPEX18*
PEX19	40 kDa farnesylated protein associated with peroxisomes	*ScPAS12*
PEX20	47kDa required for the oligomerization of thiolase for its targeting to the peroxisome.	*YlPEX20*
PEX21	Essential for targeting of proteins via PTS2.	*ScPEX21*
PEX22	Essential for peroxisomal matrix protein import, anchors the ubiquitin-conjugating enzyme, Pex4p, on the peroxisomal membrane.	*PpPEX22*

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2.5.3 Peroxisome membrane lipid acquisition, proliferation and segregation

2.5.3.1 Membrane lipid acquisition

Although much has been achieved by identifying different *PEX* genes, relatively little is known about the membrane lipid acquisition, proliferation and segregation of peroxisomes. The membrane of a peroxisome consists primarily of phosphatidyl choline and phosphatidyl ethanolamine. However, it is unclear how peroxisomes acquire lipids. Peroxisomes do not have their own biosynthetic system, and it is most probable that the lipids are acquired from the endoplasmic reticulum (ER) (reviewed in Subramani 1993). A peroxin implicated in membrane biogenesis and maintenance has been cloned in *S. cerevisiae* and was identified as *PEX3* (Baerends *et al.* 1996). The amino-terminal section of Pex3p was shown to contain ER-targeting information, suggesting that the protein may be sorted to the peroxisome via the ER (Baerends *et al.* 1996). However, the precise mechanism by which the Pex3p might help in forming the membrane is unclear.

2.5.3.2 Peroxisome proliferation

An important question concerning the proliferation of peroxisomes is whether they originate from existing peroxisomes or are formed *de novo*. Proliferation of peroxisomes was first believed to only occur by a fission or budding process from existing peroxisomes (reviewed in Subramani 1993). This fission process has only been observed in yeast cells that were undergoing rapid proliferation in response to a peroxisome inducing substrate (Veenhuis and Harder 1991). The hypothesis is that new peroxisomes cannot form unless there are pre-existing ones to spawn them. However, many yeast peroxisome biogenesis mutants have been isolated in which peroxisomes appear to be completely absent. In these strains, introduction of a wild-type copy of the defective gene causes the reappearance of peroxisomes. This apparent paradox has been explained for similar human mutant cell lines (Zellweger syndrome) by the discovery of peroxisomal membrane ghosts in the mutant cells (Santos *et al.* 1992). These ghosts are remnants of peroxisomes not visible with normal microscopy techniques. Introduction of a wild-type gene could restore to the ghosts the ability to import matrix proteins. A way to detect these peroxisome remnants was also developed by using an epitope-tagged version of peroxisome integral membrane protein and detecting remnants by immunogold labelling (Purdue and Lazarow 1995). These discoveries suggest that there must be some form of peroxisome present in the cell in order for proliferation to occur.

A gene, *PEX11*, was identified as an important factor in peroxisome proliferation from existing peroxisomes in *S. cerevisiae* (Erdmann and Blobel 1995). In this organism, the peroxisomes (induced by transfer to oleic acid media because the mutant is not able to

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grow on this media) of *pex11* mutant cells are fewer but considerably larger than those of wild-type cells. This suggests that Pex11p might be involved in proliferation of peroxisomes to set amounts. The growth defect of *pex11* cells on oleic acid therefore appears to result from the inability to segregate the giant peroxisomes to daughter cells (Erdmann and Blobel 1995). The role of Pex11p in peroxisome proliferation is confirmed by the fact the overexpression of this protein leads to a significant increase in the number of peroxisomes (Marshall *et al.* 1995). Together these results demonstrate that Pex11p is a key gene in proliferation of peroxisomes from existing peroxisomes.

There are, however, some data suggesting the opposite, i.e. that peroxisomes can form *de novo* in the absence of existing peroxisomes or peroxisome remnants (Waterham *et al.* 1993). In a study conducted by these authors, a temperature sensitive *pex* (*pex*ts) mutant was used that lacked peroxisomes at nonpermissive temperatures but had peroxisome membrane ghosts at permissive temperatures. Upon a shift to the permissive temperature, new peroxisomes were rapidly formed. Heterologous membrane proteins used to mark the remnants, which were present in the cytosol prior to the temperature shift, were not incorporated into the newly formed peroxisomes. Instead, these proteins remained unaffected in the cytosol regardless of the further peroxisome development These peroxisomes were therefore formed without involving another peroxisome or peroxisome remnant (Waterham *et al.* 1993).

A gene responsible for the development of peroxisomes *de novo* has subsequently been cloned in yeast. The *PEX16* gene was first cloned from *Y. lipolytica* and the Pex16p was identified as a peripheral protein localized at the matrix face of the peroxisomal membrane (Eitzen *et al.*1997). The *pex16* mutant lacks morphologically recognisable peroxisomes and peroxisomal proteins are mislocalized to the cytosol. Unlike other peroxins, Pex16p is synthesized in wild-type cells grown in glucose-containing media, and its levels are only modestly increased by growth of cells in oleic acid-containing media. Also, overexpression of the *PEX16* gene in oleic acid grown cells leads to the appearance of a small number of enlarged peroxisomes, which contain the normal complement of peroxisomal proteins at levels approaching those of wild-type peroxisomes (Eitzen *et al.*1997). From these results it is apparent that Pex16p has a role to play in the continuing existence of peroxisomes in cells under all growth conditions.

The role of Pex16p in *de novo* synthesis of peroxisomes was first understood when it was expressed in human cells (South and Gould, 1999). Expression of the human Pex16p results in the formation of new peroxisomes in peroxisome biogenesis disorder (PBD) cells that contain a mutated *PEX16* gene. Peroxisome synthesis and peroxisomal membrane protein import could be detected within the first 3 hours after the injection of *PEX16* into the cells and this was followed by matrix protein import. These results show that peroxisomes do not necessarily arise from division of pre-existing peroxisomes but can be

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formed *de novo* through the expression of *PEX16* (South and Gould 1999). Today, it is thought that peroxisomes arise by either of two pathways: one that involves *PEX11*-mediated division of pre-existing peroxisomes, and another that involves *PEX16*-mediated formation of peroxisomes in the absence of pre-existing peroxisomes. The reason why two pathways are present is unclear.

Two genes, *PEX10* and *PEX3*, involved in other aspects of proliferation have been cloned. In *H. polymorpha*, overexpression of Pex10p leads to a significant increase in peroxisome numbers (Tan *et al.* 1995). The Pex10p is an integral peroxisomal membrane protein. The fact that it is concentrated in the membranes of newly formed peroxisomes indicates that it is involved in the early stages of peroxisome biogenesis (Tan *et al.* 1995). The Pex10p possesses zinc binding motifs. In *P. pastoris*, point mutations in *PEX10* that change the zinc-binding conserved residues of the Pex10p C3HC4 motif obliterates Pex10p activity and reduces zinc binding, suggesting that Pex10p binds zinc *in vivo* and that zinc binding is essential for *PEX10* function (Kalish *et al.* 1995). The loss of Pex10p leads to the accumulation of peroxisomal membrane sheets and vesicles that do not have a recognisable lumen. Pex10p therefore appears to be essential in the formation of the peroxisomal lumen as well as protein translocation into peroxisomes at an early stage of biogenesis (Kalish *et al.* 1995). The human *PEX10* gene was identified based on homology using the protein sequence of the Pex10p from *H. polymorpha*. Astonishingly, the expression of the native *H. polymorpha PEX10* gene restored peroxisome biogenesis in fibroblasts from Zellweger patients of complementation group B. Patients of complementation group B had mutations in their *PEX10* gene demonstrating that these mutations are the genetic cause of this specific defect (Okumoto *et al.* 1998).

It has been suggested that peroxisome proliferation and protein import is a coupled process. In *H. polymorpha*, a sharp increase in the level of Pex3p caused the formation of numerous small peroxisomes (Baerends *et al.* 1997). The Pex3p is implicated in the biosynthesis and maintenance of the peroxisomal membrane and also in peroxisomal protein import (Baerends *et al.* 1996). Interestingly, the induction of these small peroxisomes by overexpressing Pex3p, was paralleled by a partial defect in matrix protein import. However, under conditions where excessive proliferation was repressed, protein import was normal, suggesting a coupled role for these two processes (Baerends *et al.* 1997). Indeed, a complicated process like protein import and peroxisome proliferation should be expected to be finely synchronised.

2.5.3.3 Segregation of existing peroxisomes

On glucose media, *S. cerevisiae* cells normally only contain one or two under-developed peroxisomes (reviewed in Subramani 1993). It is possible that the cell has some mechanism with which it can make sure that each daughter cell receives at least one

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peroxisome before division. In this process the role of the cytoskeleton immediately comes to mind, and indeed a study has shown that peroxisome movement is inhibited by agents that deplete ATP or by the microtubule-depolymerizing drug nocodazole (Rapp *et al.* 1996). Segregation of peroxisomes to daughter cells is however still not clearly understood.

From the data presented in this section it is apparent that Pex3p, Pex10p, Pex11p and Pex16p are the only peroxins identified to date that have a direct function in the proliferation of peroxisomes, with the latter two playing a part in the two pathways of proliferation. In future it would be interesting to see whether these proteins interact with other proteins or with each other and exactly how the machinery that controls the proliferation of the peroxisome functions.

2.5.4 Import of proteins into peroxisomes

2.5.4.1 Peroxisomal targeting signals: PTS1, PTS2 and internal signals

The peroxisomal membrane and matrix proteins are encoded by nuclear genes, synthesized on free ribosomes and released in the cytosol before they are imported into the peroxisome (reviewed in Lazarow and Fujiki 1985). The proteins are post-translationally modified and imported by a process that requires ATP and is aided by hsp70-class cytoplasmic chaperones (Walton *et al.* 1994). This includes all the peroxisome matrix enzymes involved in the β-oxidation pathway. Also included are peroxisomal membrane proteins like the fatty acid transporter proteins Pat1 and Pat2 (Hettema *et al.* 1996). The import of these peroxisomal proteins requires that the proteins contain a peroxisomal targeting signal that will direct it to the peroxisome.

The first peroxisomal targeting signal (PTS) was discovered in firefly luciferase and named PTS1 (Gould *et al.* 1988). The signaling peptide Serine-Lysine-Leucine (SKL) resides at the end of the carboxy terminus of the protein and is required for peroxisomal import. When the PTS1 is attached to the carboxyl terminus of non-peroxisomal proteins, these proteins are directed to the peroxisome (Gould *et al.* 1988). In order to identify the variability of the conserved protein sequence of PTS1, different permutations of the PTS1 were tested for their targeting efficiency. Variable targeting efficiencies were detected (Swinkels *et al.* 1992). The consensus sequence identified consists of a small amino acid at the first position, then a basic amino acid at the second position and a leucine or methionine at the last position. A corresponding protein sequence is sufficient for effective targeting to the peroxisome (Swinkels *et al.* 1992). An example of a yeast protein targeted

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to the peroxisome matrix with a PTS1 signal is the β-oxidation enzyme acyl-CoA oxidase (Pox1p) in *P. pastoris* (Koller *et al.* 1999b).

All peroxisomal proteins targeted to the peroxisome do not necessarily have the PTS1 signal. A second peroxisomal targeting sequence was discovered in a rat peroxisomal thiolase, one of the enzymes responsible for β-oxidation. The PTS2 sequence is located at the N-terminal extremity of the protein and has the consensus sequence Arg-Leu-X5-His/Gln-Leu (Swinkels *et al.* 1991). In *S. cerevisiae*, a PTS2 sequence is present at the N-terminus of the peroxisomal 3-ketoacyl-CoA thiolase. *In vivo* expression has shown that the amino-terminal 16 amino acids of this thiolase are necessary and sufficient for targeting it to the peroxisome (Glover *et al.* 1994a).

Several internal peroxisome signal sequences have also been described. For example, the acyl-CoA oxidase in *S. cerevisiae* in addition to the PTS1 signal already present, has an internal signal sequence that can target it to the peroxisome (Small *et al.* 1988). In another case, proteins have been identified that contain two signals diverting it to two different compartments. This is the case for the carnitine acetyltransferase in *S. cerevisiae*, a protein with a mitochondrial targeting signal (MTS) at the N-terminus and a PTS1 sequence at the C-terminus (Elgersma *et al.* 1995). In addition to this, deletion of both motifs revealed the presence of an internal peroxisomal targeting sequence. Import of the carnitine acetyltransferase via this internal signal was shown to be dependent on Pex5p, a protein that is also required for the import of PTS1 proteins (Elgersma *et al.* 1995).

2.5.4.2 Receptors for peroxisomal proteins: Pex5p and Pex7p

In order to import proteins with PTS signals into peroxisomes, these signals have to be recognised by components of the import machinery. These receptors have been identified for both PTS1 and PTS2 (reviewed in Rachubinski and Subramani 1995). The first PTS1 receptor was described in *P. pastoris* as the product of the *PEX5* gene, Pex5p (Terlecky *et al.* 1995). It was shown that a *pex5* mutant is deficient in the import of proteins containing PTS1, but not PTS2. Pex5p binds proteins ending in the conserved SKL sequence of PTS 1 with high affinity and specificity *in vitro* (Terlecky *et al.* 1995). This import deficiency caused by a defective Pex5p in *P. pastoris* is the same defect characteristic of cells from patients with the lethal human peroxisomal disorder Zellweger syndrome (McCollum *et al.* 1993). A human homologue of the *P. pastoris PEX5* gene, the *PXR1* gene, was cloned based on homology of the amino acid sequence of the yeast Pex5p to human proteins. Similar to the yeast Pex5p, the human Pex5p contains seven copies of the tetratricopeptide (TPR) repeat motif that binds proteins containing the carboxy terminal SKL sequence. The functional human *PEX5* gene rescues the PTS1 import defect of fibroblasts of one complementation group of patients with Zellweger syndrome. The cell-

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lines of this complementation group also contain mutations in *PEX5*. It is clear that *PEX5* is one of the defective genes responsible for this disease (Dodt *et al.* 1995).

The subcellular localization of the Pex5p receptor would cast some light on how it may contribute to the import of PTS1 containing proteins into the peroxisome. However, this has been a matter of controversy over the last few years, with studies in different model organisms suggesting different mechanisms. Pex5p has been found to be: (a) both peroxisome associated and in the cytosol; (b) exclusively in the peroxisomal matrix (c) mainly associated with the peroxisomal membrane (reviewed in Hettema *et al.* 1999). These various localizations lead to different speculative models of PTS1 receptor functioning. The *P. pastoris* Pex5p is reportedly tightly associated with the cytoplasmic side of the peroxisomal membrane (Terlecky *et al.* 1995). This would suggest that the receptors act on the outside of the peroxisomal memrane and then binds PTS1 containing proteins prior to translocation (**Fig. 2.8A**). In contrast to this, the *H. polymorpha* Pex5p is found both on the outside of the peroxisomal membrane and in the cytosol (Van der Klei *et al.* 1995). Human Pex5p is found primarily in the cytosol and only a small amount is present on the outside of the peroxisomal membrane (Dodt *et al.* 1995). Differential localization as in this case suggests a cycling model in which the receptors shuttle between the cytosol and the peroxisomal matrix. Through this process it would deliver the PTS1 containing protein with each cycle to the peroxisomal matrix (**Fig. 2.8B**). On the other hand, the *Y. lipolytica* Pex5p is associated primarily with the inner surface of the peroxisomal membrane (Szilard *et al.* 1995). This might suggest a model where the receptor acts intra-peroxisomal and binds PTS1 proteins as they emerge through the peroxisomal membrane, pulling them into the organelle (**Fig. 2.8C**).

Presently, the general feeling among researchers is that a cycling model is the most probable. Experimental evidence supporting the cycling of the Pex5p receptor between the cytosol and the peroxisome was obtained in human fibroblast. Under conditions that make peroxisomal protein import difficult (low temperature and ATP depletion), Pex5p was partially trapped at the surface of the peroxisome and Pex5p returned to the cytoplasm after the conditions were restored to normal. The Pex5p was again trapped at the surface of the peroxisomal membrane when the conditions were changed back to that of not favouring import (Dodt and Gould 1996).

A PTS2 receptor was cloned when it was discovered that a *pex7* mutant of *S. cerevisiae* was unable to import thiolase, a protein containing the PTS2 sequence. The *PEX7* gene was cloned by complementation of the mutant strain and identified as a member related to the beta-transducin (WD-40) family of proteins (Marzioch *et al.* 1994). The same phenotype seen for the yeast *pex7* mutant is also found in patients with the peroxisomal disorder rhizomelic chondrodysplasia punctata (RCDP) suggesting that this disease is due to a defective PTS2 import system (Slawecki *et al.* 1995).

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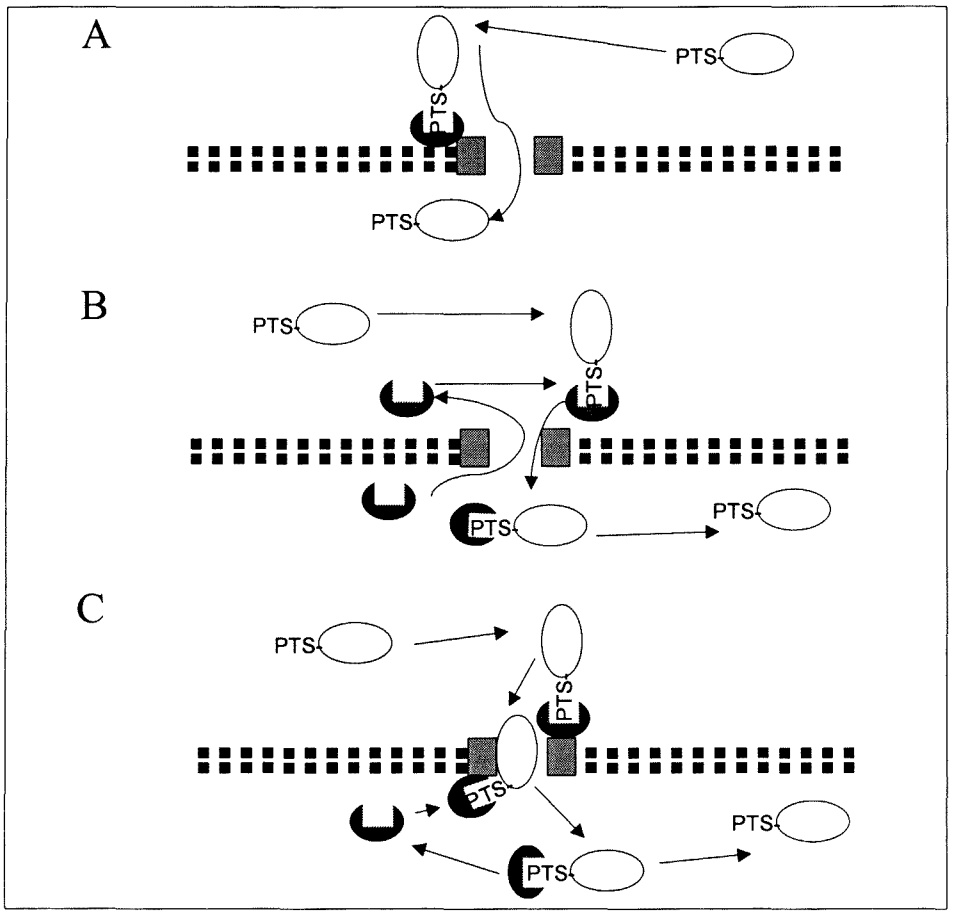

Fig. 2.8. Proposed models of protein import into peroxisomes based on reported subcellular locations of the putative receptor proteins. (A) Proteins bind to the PTS receptors on the outer surface of the peroxisomal membrane and are then transferred to the translocation apparatus for import into the matrix. (B) Proteins bind to PTS receptors in the cytosol and are translocated into the peroxisomal matrix as a complex. In the matrix receptors dissociate from the proteins and are recycled back to the cytoplasm. (C) Proteins are bound by the PTS receptors located in the peroxisomal matrix as they emerge through the peroxisomal membrane and facilitate their further translocation through the import apparatus (adapted from Waterham and Gregg 1996).

The human orthologue of *PEX7* was subsequently cloned and it was demonstrated that this is the defective gene in RCDP (Purdue *et al.* 1997). Patients with RCDP have peroxisomes characterized by deficiencies in several peroxisomal enzymes, including the

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enzymes of plasmalogen biosynthesis. Expression of human *PEX7* in RCDP cells rescues PTS2 targeting and restores activity of some of the plasmalogen biosynthesis enzymes (Purdue *et al.* 1997). In *S. cerevisiae*, the function of Pex7p was confirmed by the two-hybrid assay, where a strong interaction was observed between Pex7p and thiolase (Zhang and Lazarow 1996). This interaction was destroyed by deleting the first 16 amino acid residues (the part that contains the PTS2 targeting signal) of thiolase. An oligopeptide containing the first 16 amino acid residues of thiolase was sufficient for the binding of Pex7p. Studies on Pex7p itself showed that deletion of residues 6-55 from native Pex7p resulted in a cytosolic location and a loss of function. Thus the NH_2-terminal 56-amino acid residues of Pex7p are essential for peroxisomal targeting (Zhang and Lazarow 1996). Also, the Pex7p is found in peroxisomes whether thiolase is expressed or not. This suggest that Pex7p is an intra-peroxisomal receptor for PTS2-containing proteins, thus supporting an intra-peroxisomal receptor model (**Fig. 2.8C**) (Zhang and Lazarow 1996). As in the case of the PTS1 receptors there is, however, controversy surrounding the localization of the PTS2 receptor. In other studies, the Pex7p was identified as being located on the outer surface of the peroxisomes in the presence of thiolases but to be located in the cytosol in the absence thereof (Marzioch *et al.* 1994). These data would rather suggest a cycling model for the PTS2 receptor function (**Fig. 2.8B**). In this model, Pex7p picks up a PTS2-containing protein in the cytoplasm and delivers it to the peroxisomal membrane. At this stage the Pex7p and ligand complex might either dissociate at the peroxisomal membrane with only the ligand being translocated and Pex7p returning back into the cytoplasm, or the ligand and Pex7p may be co-imported into peroxisomes where they dissociate, with Pex7p being exported back into the cytoplasm.

Recent studies in *P. pastoris* have added more data supporting the cycling model (Elgersma *et al.* 1998). Using a new screening procedure for the isolation of peroxisomal import mutants in this organism, a *pex7* mutant was isolated that cannot import proteins containing a PTS2 into the peroxisome. As in the case of its *S. cerevisiae* homologue, this particular Pex7p interacted with the PTS2 in the two-hybrid system, suggesting that Pex7p functions as a receptor. It was also demonstrated that Pex7p is both cytosolic and intra-peroxisomal, supporting the cycling model (Elgersma *et al.* 1998).

Although controversy exists regarding the model for receptor function, it seems that the cycling model is the most probable for both Pex5p and Pex7p function. However, this hypothesis remains to be proven.

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2.5.4.3 Receptor associated proteins: Docking and translocation

It appears that the translocation of proteins into peroxisomes is based on two pathways involving PTS1 and PTS2. To better understand these pathways it is important to identify additional proteins involved and their functions. This has led to the cloning of peroxins involved in translocation through their interaction with the PTS1 and PTS2 receptors, Pex5 and Pex7, respectively. Some of these peroxins also interact with each other and also with other peroxins that are not directly interacting with either Pex5p or Pex7p. Based on the receptor cycling model, three events can be distinguished that contribute to the translocation of peroxisomal matrix proteins across the membrane (reviewed in Hettema *et al*. 1999). First, the PTS-containing protein complexed to its receptor has to bind to a docking protein complex. Second, the PTS-containing protein has to be delivered to the translocation machinery and translocated across the membrane. Third, the receptor has to be shuttled back to the cytoplasm. Candidate proteins involved in some of these processes have subsequently been identified and are discussed in the following sections.

One of these candidate peroxins is Pex13p, which was first identified in *S. cerevisiae* (Erdmann and Blobel 1996). This integral membrane protein acts as a docking system for PTS-containing proteins. Pex13p is a 43-kD protein containing a src homology 3 (SH3) domain near its carboxyl terminus. This specific domain interacts directly with the Pex5p receptor, but the SH3 domain itself does not interact with the Pex7p receptor. The fact that Pex13p is located on the outside of the peroxisomal membrane suggest that it acts as a docking protein on the surface of the peroxisome, binding protein-receptor complexes in the protein import process (Erdmann and Blobel 1996). The human disease neonatal adrenoleukodystrophy (NALD), a disease that is usually associated with partial defects in the import of peroxisomal matrix proteins with PTS1 and PTS2 targeting signals, is a milder form of a peroxisome biogenesis disorder (PBD). It was demonstrated that skin fibroblasts from a patient with NALD displayed defects in the import of multiple peroxisomal matrix proteins (Liu *et al*. 1999). The expression of the human *PEX13*, however, restored peroxisomal matrix-protein import in these cells. As expected, site directed mutations of the conserved position of the *PEX13* SH3 domain reduced its import activity. This demonstrated that mutations in the human *PEX13* gene are most probably responsible for NALD at least in some patients.

Another docking complex protein found to interact directly with the PTS receptors was identified in *S. cerevisiae* as Pex14p (Albertini *et al*. 1997). Like Pex13p, this protein is attached to the outer face of the peroxisomal membrane suggesting a protein-receptor docking system. Interestingly, Pex14p interacts with both the PTS1 and PTS2 receptors. These interactions suggest that the PTS1 and PTS2 import pathways are not independent, but overlap and that Pex14p might be at the point of convergence for these two pathways.

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This hypothesis is supported by the fact that Pex14p also interacts with Pex13p, the docking protein for the PTS1 receptor.

The interaction of Pex13p and Pex14p was further confirmed when it was demonstrated that Pex13p interacts indirectly with Pex7p (Girzalsky *et al.* 1999). This interaction occurs only in the absence of Pex14p. In cells that lack Pex14p, Pex13p efficiently co-immunoprecipitates with Pex7p and it also interacts with Pex7p in the yeast two-hybrid system. Thus Pex7p and Pex13p functionally interact during PTS2-dependent protein import into peroxisomes (Girzalsky *et al.* 1999). It was also shown that this interaction with Pex14p occurs via the SH3 domain of Pex13p (Albertini *et al.* 1997). However, the SH3 domain of Pex13p may not provide the only binding site for Pex14p at the peroxisomal membrane (Girzalsky *et al.* 1999).

There are components of the peroxisomal translocation machinery that do not interact directly with PTS receptors Pex5p and Pex7p, but seem to act as part of a docking complex. The Pex17p is one of these components and interacts with Pex14p, the docking protein for Pex7p (Huhse *et al.* 1998). The *pex*17 null mutants fail to import matrix proteins into peroxisomes via both PTS1 and PTS2-dependent pathways. It has been shown that Pex17p directly interacts with Pex14p, the proposed point of convergence for the two peroxisomal targeting signal (PTS)-dependent import pathways. It also indirectly interacts with Pex5p and Pex7p, the PTS1 and PTS2 receptors respectively. These interactions of Pex17p with Pex5p and Pex7p requires Pex14p, suggesting that Pex17p and Pex14p form a complex. This hypothesis has been strengthened by immunoprecipitation experiments, which showed that Pex14p and Pex17p co-precipitate with both PTS receptors in the absence of Pex13p (Huhse *et al.* 1998).

The above mentioned results suggest that a protein-receptor complex is transferred to the peroxisome where it is recognised by a complex which comprises of Pex13p, Pex14p and Pex17p. The receptor then releases the cargo, which is transported into the peroxisome. The function of the Pex13p, Pex14p and Pex17p may not be limited to the creation of a docking site alone, but could also be involved in the translocation process. The presence of these multiple PTS-receptor-binding peroxins located at the peroxisomal membrane suggest the existence of an import cascade. Successive interactions of a PTS receptor with peroxins at the peroxisomal membrane might induce conformational changes in the PTS receptor allowing for the release of the matrix protein and recycling of the receptor. For the PTS2 pathway this cascade might also involve Pex18p and Pex21p (Purdue *et al.* 1998). A model of this complex is represented in **Fig. 2.9**.

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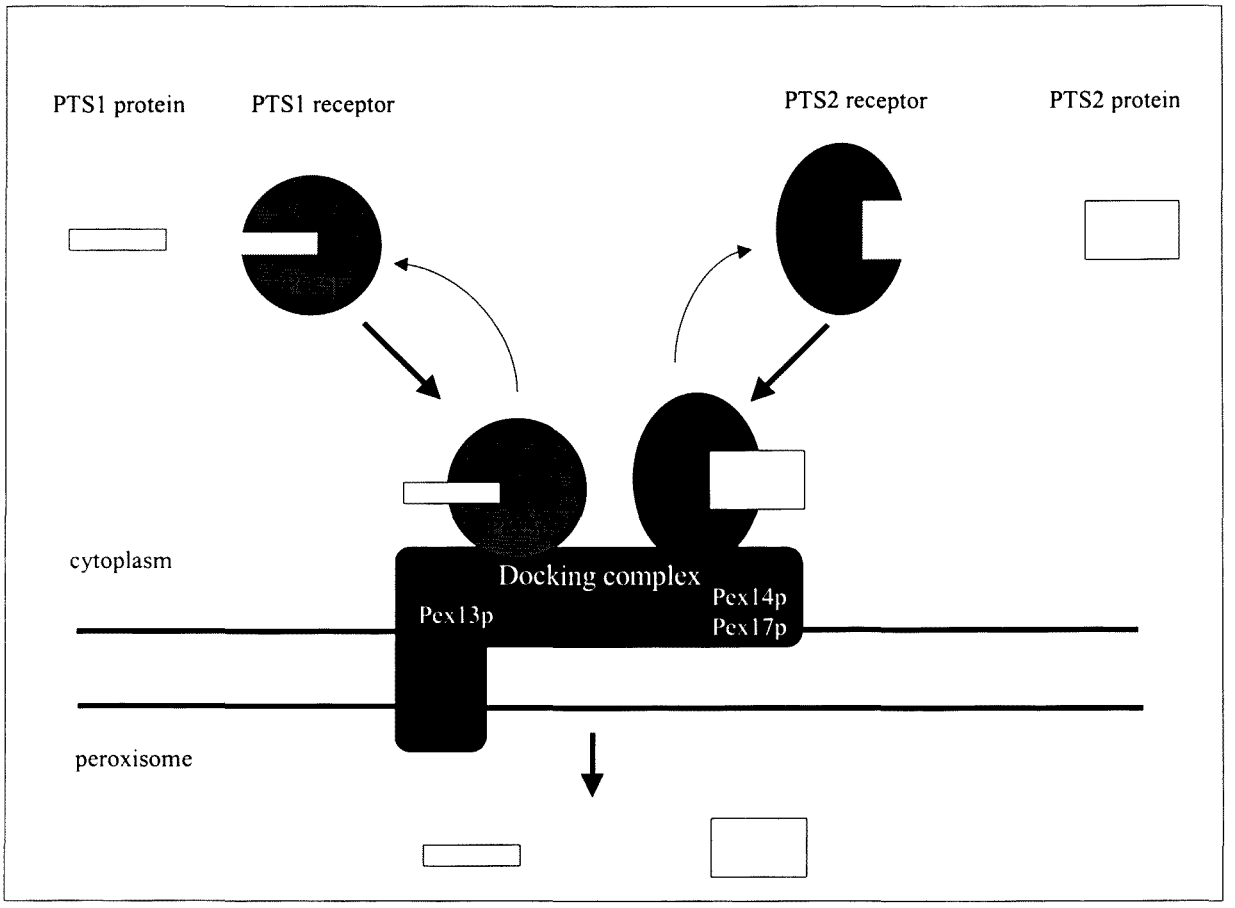

Fig. 2.9. Model for the two pathways of peroxisomal protein targeting. The PTS receptors Pex5p and Pex7p recognise PTS-containing proteins in the cytoplasm. The receptor complexes bind to the peroxisomal membrane docking complex that consist of Pex13p, Pex14p and Pex17p (adapted from Hettema *et al.* 1999).

The additional peroxins, Pex18p and Pex21p, are required for protein targeting exclusively via the PTS2 pathway and were first identified in *S. cerevisiae*. The Pex18p and Pex21p each interact specifically with Pex7p both in two-hybrid analyzes and *in vitro*. In cells lacking both Pex18p and Pex21p, the Pex7p receptor remains cytosolic and PTS2 targeting is completely absent. These results suggest that Pex18p and Pex21p function in directing Pex7p to the peroxisome (Purdue *et al.* 1998). The localization of Pex18p (mainly cytosolic, with a minor fraction associated with the cytoplasmic face of the peroxisomal membrane) coincides with such a role.

An additional protein involved in peroxisomal import is Pex4p. The Pex4p protein has high homology with the ubiquitin-conjugating (UBC) proteins, which transfer an ubiquitin group to a target protein. It is the first member of this family of proteins discovered that plays a role related to peroxisomes (Wiebel *et al.* 1992). *PEX4* has been cloned from *P. pastoris*.

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Substitution of the conserved active-site cysteine residue with a serine residue abolished Pex4p function as expected for an ubiquitin-conjugating protein. A function of Pex4p in *H. polymorpha* has recently been uncovered. It was shown that Pex4p is required for efficient functioning of the PTS1 (Van der Klei *et al.* 1998). The *pex4* strain had a specific defect in import of peroxisomal matrix proteins containing PTS1 and also accumulated the Pex5p receptor inside the peroxisome. This defect was suppressed by the overproduction of the Pex5p receptor. These results suggested that Pex4p perform a very unique function by mediating recycling of the Pex5p receptor (Van der Klei *et al.* 1998). In *P. pastoris,* a peroxin, Pex22p, interacting with Pex4p was recently discovered (Koller *et al.* 1999a). Data in this study suggested that the role of Pex22p is to anchor Pex4p to the peroxisomal membrane since in strains lacking Pex22p, Pex4p is cytosolic and unstable.

A peroxin involved in the early stages of protein import is Pex19p, an oleic acid-inducible, farnesylated protein first identified in *S. cerevisiae* (Gotte *et al.* 1998). In *pex19* mutant cells, morphologically detectable peroxisomes are absent and mislocalization of peroxisomal matrix proteins with PTS1 and PTS2 occurs. Pex19p was shown to interact with Pex3p, a peroxisomal matrix protein implicated in the synthesis of the peroxisomal membrane and in peroxisomal proliferation (Baerends *et al.* 1996; Gotte *et al.* 1998). In *P. pastoris*, Pex19p also interacts with Pex3p, similar to the results obtained for *S. cerevisiae* (Snyder *et al.* 1999). Interestingly, Pex19p also interacts with Pex10p, an integral membrane protein that is concentrated in the membranes of newly formed organelles (Tan *et al.* 1995; Snyder *et al.* 1999). Two-hybrid analysis demonstrated that the amino-terminal 42 amino acids of Pex19p interact with the carboxyl-terminal 335 amino acids of Pex3p in *P. pastoris*. The extreme carboxyl terminus of Pex19p is required for interaction with the amino-terminal 380 amino acids of Pex10p (Snyder *et al.* 1999). Because of the involvement of Pex3p and Pex10p, it is suggested that Pex19p functions in the import of proteins at an early stage of peroxisome biogenesis.

Pex12p is one of the peroxins that contains a C_3HC_4 zinc binding domain which is essential for its biological activity. In *P. pastoris,* mutant cells lacking the *PEX12* gene mislocalized both PTS1-and PTS2-containing proteins to the cytoplasm. Most of the peroxisomes from *pex12* cells migrated to a much lower density than translocation-competent cells. The *pex12* peroxisomes also had an abnormally low ratio of matrix: membrane distribution of proteins. The Pex12p seems to play an important role in matrix protein translocation across the peroxisome membrane (Kalish *et al.* 1996). The human *PEX12* gene was cloned based on homology to yeast Pex12p. The expression of the *PEX12* gene restored peroxisomal protein import in fibroblasts from PBD patients of complementation group 3 (Chang *et al.* 1997).

Taken together, the data discussed in the section above suggest the existence of an extended shuttle system. In this system, the import receptors Pex5p and Pex7p bind cargo

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proteins in the cytosol, dock to a specific complex consisting of Pex13p, Pex14p and Pex17p at the periphery of the peroxisomal membrane, followed by entry into the peroxisome and releasing of there cargo in the lumen. The receptors then shuttle back to the cytoplasm. Although there is no concrete experimental evidence to support this model, it does coincide with the fact that peroxisomes are able to import folded and oligomeric proteins, an aspect that will be discussed in a later section. It is for future research to establish the exact mechanism of protein translocation and all the peroxins involved.

2.5.4.4 Other proteins associated with protein import

The Pex1p and Pex6p proteins belong to ATPases associated with the diverse cellular activities (AAA) family of ATPases (Kunau *et al.* 1993). Pex1p and Pex6p actually belong to a subfamily of these ATPases containing two ATP binding sites. Other members of this sub-family, CDC48 and NSF/SEC18, are both involved in membrane fusion (reviewed in Mellaman 1995). Recently it has been shown in *P. pastoris* that these two proteins interact with each other in an ATP dependent manner and that they form part of subcellular membranous structures other than of peroxisomes (Faber *et al.* 1998). It is argued that the interaction between Pex1 and Pex6 plays a part in regulating peroxisome biogenesis. This interaction has also been shown for *H. polymorpha* where the fact that Pex1p and Pex6p physically and functionally interact *in vivo* was demonstrated by two-hybrid analysis. The overexpression of *PEX6* results in aberrant peroxisome assembly and defects in peroxisomal matrix protein import. However, overexpression of *PEX1* did not lead to similar detrimental effects. In cell fractionation studies, Pex1p and Pex6p co-sedimented with peroxisomal membrane proteins, indicating its peroxisomal location. These results and the fact that co-overproduction of Pex1p rescues the protein import defect caused by Pex6p overproduction suggest that Pex1 and Pex6p function in a protein complex associated with the peroxisomal membrane (Kiel *et al.* 1999). The human *PEX1* gene has been identified based on homology using the *S. cerevisiae* Pex1p sequence. The human *PEX1* gene encodes a 147-kD member of the AAA protein family. Expression of human *PEX1* rescued the mutant cells from the peroxisome biogenesis defect present in human fibroblasts of complementation group 1 (CG1), which causes severe disease in humans (Portseffen *et al.* 1997).

An interesting protein that was identified in *H. polymorpha* is Pex8p (Waterham *et al.* 1994). The gene was cloned by functional complementation of a *pex8* mutant, which was impaired in the import of peroxisomal matrix proteins. Further analysis of the Pex8p protein revealed that it contains both a carboxy- (PTS1) and an amino-terminal (PTS2) peroxisomal targeting signal. The low abundance of Pex8p in the peroxisomal matrix and the existence of structures representing immature peroxisomes suggest that the import of Pex8p into peroxisomes is a prerequisite for the import of additional matrix proteins. Pex8p

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could therefore play a regulatory function on peroxisomal protein import (Waterham *et al.* 1994).

In *Y. lypolytica*, functional complementation of a *pex9* mutant with a genomic library identified the *PEX9* gene that restores growth of *pex9* mutants on oleic acid, import of catalase and peroxisomal matrix enzymes into peroxisomes and formation of wild type peroxisomes (Eitzen *et al.* 1995). Very little is known about this peroxin. Pex9p shows no similarity to any known protein The *PEX9* gene encodes Pex9p, a hydrophobic polypeptide of approximately 42-kDa that is a peroxisomal integral membrane protein. (Eitzen *et al.* 1995).

Another unique peroxin is Pex15p (Elgersma *et al.* 1997). The *PEX15* gene is required for peroxisome biogenesis in *S. cerevisiae* and is a peroxisomal membrane protein. Interestingly, the overexpression of Pex15p not only results in impaired peroxisome assembly due to defective protein import, but also causes significant proliferation of the endoplasmic reticulum (ER) membrane. Pex15p has a peroxisomal membrane targeting signal and an ER targeting signal that overlap. This suggests that Pex15p may be targeted to peroxisomes via the ER, or to both organelles (Elgersma *et al.*1997).

The Pex20p has a unique function in that it is involved in the import of the PTS2-containing thiolase through the formation of a heterotetrameric complex containing two thiolase proteins and two Pex20p proteins (Titorenko *et al.* 1998). Pex20p is mostly cytosolic with very small amounts located in the peroxisome. The complex has to form in order for import to take place. Thiolase import is also dependent on Pex7p (Marzioch *et al.* 1994). Although the Pex20p seem to play a similar role to the Pex7p in transporting thiolase, it does not exhibit homology with Pex7p. The mechanism by which the thiolase is transported into the peroxisome seems to be based on the Pex20p-thiolase heterotetrameric complex locating to the peroxisome followed by the translocation of the thiolase homodimer into the peroxisomal matrix. The Pex20p monomers are then released back to the cytosol. This allows for a new cycle of binding-oligomerization-targeting-release for Pex20p and thiolase to proceed (Titorenko *et al.* 1998).

Although genetic and biochemical approaches have resulted in the identification of a large amount of components involved in peroxisomal protein import, the functioning of these components still has to be established. Novel genetic screens could also identify more components, whereas experimental approaches based on *in vivo* import assays could help elucidate the dynamics of the import process. Additionally, the peroxisomes have to be studied in more detail on the structural level to understand the extent of protein-protein interaction and how these interactions result in conformational changes.

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2.6 IMPORT OF FOLDED PROTEINS INTO PEROXISOME

Contrary to the dogma that protein import into organelles requires unfolding of the proteins prior to import, as is the case for mitochondria, peroxisomes can import proteins in a folded form (McNew and Goodman 1994). Convincing evidence supporting this hypothesis was given when it was shown in *S. cerevisiae*, that when thiolaseΔPTS2 is co-synthesized with full-length thiolase, approximately 50% of thiolaseΔPTS2 co-fractionates with the full-length thiolase to fractions enriched for peroxisomes. The active conformation of native thiolases is homodimeric. This indicates that the thiolase complex is transported and not the thiolase alone. The presence of the complex in the peroxisome was demonstrated by the fact that thiolaseΔPTS2 is protected in the peroxisome from the action of external proteases (Glover 1994b). The final proof for the import of folded proteins into peroxisomes came when it was demonstrated that colloidal gold particles covered in HSA-PTS1 (human serum albumin, cross-linked to PTS1 peptides) could be imported into peroxisomes (Walton *et al.* 1994).

The fact that proteins can be transported into peroxisomes as dimers or multimers has opened up new views on how proteins can be targeted to the peroxisome without containing PTS1 or PTS2. It is possible that these proteins could associate with PTS-containing proteins and then be co-imported into the peroxisome. In this case, the dimerisation domain could be mistakenly identified as an internal targeting signal. Associations between two peroxisomal enzymes, isocitrate lyase and malate synthetase, where only one has a PTS signal, has already been demonstrated (Beeckmans *et al.* 1994).

It is clear that the import machinery of peroxisomes is different from other organelles. How does protein translocation take place? One view is that peroxisomes might have large pores but that these are transient (assembles for translocation and then rapidly disassembles) since these pores have not been detected in peroxisomes (McNew and Goodman 1994). Another possibility is that the proteins can enter the peroxisome by an invagination process with the resulting internal vesicles being degraded and releasing their contents (McNew and Goodman 1994). To date, the mechanisms of peroxisomal protein translocation remains to be elucidated.

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2.7 DEGRADATION OF PEROXISOMES

In order for cells to adapt to changing growth conditions, a rapid mechanism of assembly and disassembly of the organelles has to exist. The development of peroxisomes is a specific adaptation to metabolic needs (reviewed in Lazarow and Fujiki 1985). In the methylotrophic yeast *H. polymorpha*, peroxisomes are induced upon shift to methanol substrates (Veenhuis and Harder 1991). When these cells are shifted back to peroxisome non-inducing substrates like glucose, peroxisomes are specifically degraded in a process that resembles autophagy (Veenhuis *et al.* 1983). In this process, layers of membranes are formed around the peroxisome; this structure is called an autophagosome. The endoplasmic reticulum might be responsible for the formation of these membranes (Dunn 1990; Ueno *et al.* 1991). These autophagosomes fuse with the vacuoles containing enzymes that degrade the peroxisome (Veenhuis *et al.* 1983). The autophagy process has also been shown to exist in *S. cerevisiae* and *P. pastoris* (Tuttle and Dunn 1995; Chiang 1996). Interestingly, two distinct autophagy processes take place in *P. pastoris*. After growth on methanol, cells shifted to glucose had their peroxisomes degraded in a microautophagy process. The glucose-induced pathway invokes the engulfment and degradation of clusters of peroxisomes by finger-like protrusions of the vacuole in a process analogous to microautophagy. A shift of methanol grown cells to ethanol resulted in degradation via a macroautophagy process as described for *H. polymorpha*. Peroxisomes are sequestred into autophagosomes by wrapping membranes, which then fuse with the vacuole (Tuttle and Dunn 1995). Subsequently, mutants defective in microautophagy have been isolated as *gsa* and *pag* mutants in *P. pastoris* (Tuttle and Dunn 1995; Sakai *et al.* 1998). Further work needs to be be done in order to identify all the molecular components involved in peroxisome degradation.

2.8 CONCLUSION

Although our understanding of peroxisomes lags behind those of other organelles, the past decade has witnessed major advances in this field. This has great implications for man where a wide variety of genetic disorders is caused by loss of peroxisome function. Research on yeast model systems has contributed to a better understanding of peroxisome function and biogenesis. These studies have given insight into the molecular basis of most of the peroxisome biogenesis disorders. In addition, many human genes defective in peroxisome biogenesis have been cloned based on homology with yeast counterparts.

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Basic questions regarding the biogenesis of peroxisomes remain to be answered. The process of lipid acquisition, proliferation and segregation still remains enigmatic, although some components thereof have been identified. This is also true for matrix protein import where some of the components of the docking and translocation system have been identified but the mechanism of action is still unclear. These gaps in our knowledge have to be filled if we intend to have a full understanding of the peroxisome. This will require novel genetic screening methods to identify more components involved. The structure and interaction of the known peroxins needs to be studied in depth to identify their exact cellular function. *In vivo* import studies of peroxisome matrix proteins will also cast additional light on the mechanism of transport across the peroxisomal membrane.

Research will unveil the secrets of the peroxisome. Hopefully, our understanding of this intriguing organelle will not only add to our knowledge of the cell, but also help in developing effective therapies for patients with defective peroxisomes.

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CHAPTER 3

RESEARCH RESULTS

Selection of mutants affected in genes required for carnitine-dependent activities in *Saccharomyces cerevisiae*: Yat1p is an essential component in a carnitine-dependent strain

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RESEARCH RESULTS

Selection of mutants affected in genes required for carnitine-dependent activities in *Saccharomyces cereviseae*: Yat1p is an essential component in a carnitine-dependent strain

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ABSTRACT

In yeast, the peroxisome is the sole site for β-oxidation of fatty acids. The peroxisomal and mitochondrial membranes, however, are impermeable to the catabolic product of this process, acetyl-CoA. The generation of energy from fatty acids therefore requires the transfer of either acetyl-CoA equivalents in the form of acetylcarnitine or intermediates of the TCA cycle from the peroxisomes to the mitochondria to fuel energy production. Intermediates of the TCA cycle, in particular C_4 compounds like malate or succinate, are indeed synthesized from acetyl-CoA in the peroxisome through the glyoxylate cycle. We have devised a strategy for the isolation of genes encoding proteins with L-carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*. Our data show that the disruption of the *CIT2* gene, which encodes the peroxisomal citrate synthetase, the first enzyme of the glyoxylate cycle, results in a strain that is dependent on the presence of L-carnitine in the growth substrate when the yeast is grown on oleic acid as sole carbon source. In this strain, only the carnitine-dependent transfer of acetyl-CoA equivalents can sustain energy production. Our strategy was therfore based on the random mutagenesis of the *CIT2* disrupted strain, followed by the selection of mutants unable to grow on oleic acid as sole carbon source, even in the presence of L-carnitine. In order to eliminate mutants affected in genes required for carnitine-independent fatty acid catabolism, all selected strains were transformed with *CIT2*, therefore re-establishing the glyoxylate cycle. Only those mutants not affected in peroxisome biogenesis and β-oxidation would be able to grow on oleic acid. Seven mutants corresponding to this phenotype were selected. Complementation of one of these mutants with a *S. cerevisiae* genomic library led to the cloning of the gene *YAT1*, that codes for the presumptive carnitine acetyltransferase of the outer-mitochondrial membrane. This indicated that, in the absence of the glyoxalate cycle, the enzyme is required for growth on oleic acid as sole carbon source even in the presence of L-carnitine.

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3.1 INTRODUCTION

L-Carnitine is a compound used in all eukaryotic cells and is required for the transfer of activated short- and long-chained acyl-groups across intracellular membranes. In mammalian cells, this role mainly consists of the transfer of long-chained fatty acids into the mitochondria for β-oxidation. It is also involved in the transport of activated medium- and short-chain organic acids from the peroxisome to the mitochondria, a process referred to as the carnitine shuttle (reviewed in Bieber 1988). In humans, carnitine deficiency is at the origin of a number of diseases. In most cases, the deficiencies are caused by genetic defects and are characterized by low levels of carnitine in either the serum or in tissues (reviewed in Pons and De Vivo 1995). In addition, carnitine has been shown to have positive therapeutic effects in the treatment of patients with diseases like AIDS, diabetes and Alzheimer's (De Simone *et al.* 1993; Keller *et al.* 1998; Carta *et al.* 1993).

In *S. cerevisiae*, L-carnitine plays a role in the transfer of activated acetyl-groups from the peroxisome to the mitochondria. This shuttle relies on the activity of carnitine acetyltransferases (CAT) (reviewed in Bieber 1988). These enzymes catalyze the transfer of the activated acetyl group of acetyl-CoA to carnitine to form acetylcarnitine and also catalyze the reverse reaction. Two genes encoding carnitine acetyltransferase have been identified in yeast. The first, *CAT2*, encodes the carnitine acetyltransferase that is localized to the inner-mitochondrial membrane and the peroxisomal membrane (Kispal *et al.* 1993; Elgersma *et al.* 1995). The second, *YAT1*, encodes a carnitine acyltransferase located in the outer-mitochondrial membrane. Cat2p is responsible for about 95% of the total CAT activity in *S. cerevisiae*, while the remaining 5% are due to the activity of Yat1p (Schmalix and Bandlow 1993)

In *S. cerevisiae*, peroxisomes are required for growth on fatty acid as sole carbon source since they are the sole site for β-oxidation, a pathway needed for the catabolism of fatty acids (Kunau *et al.* 1988). When grown on fermentable carbon sources, no clearly identifiable peroxisomal structures can be detected in *S. cerevisiae*, but when grown on fatty acids like oleic acid, a strong induction of peroxisomes occurs (Veenhuis *et al.* 1987). The peroxisomal membrane, however, is impermeable to the catabolic product of β-oxidation, acetyl-CoA, which is required in the mitochondrion where it is used in the tricarboxylic acid cycle (TCA) for the production of energy and for gluconeogenesis. In *S. cerevisiae*, two pathways for further metabolic conversion of peroxisomally produced acetyl-CoA have been described (Van Roermund *et al.* 1995). The first pathway relies on the carnitine acetylcarnitine shuttle which allows the transfer of the activated acyl group from acetyl-CoA to a carnitine molecule, resulting in the formation of free CoA and an acetylcarnitine ester. The acetylcarnitine is then shuttled to the mitochondrion where the acyl group is transferred back to CoA, allowing metabolism via the TCA cycle. The second

pathway utilizing peroxisomal acetyl-CoA is the glyoxylate cycle, which leads to the formation of the C_4 compound succinate, which is a TCA cycle intermediate (Van Roermund *et al.* 1995). This compound is transported into the mitochondrion for metabolism in the TCA cycle or other metabolic pathways. The existence of two pathways, the carnitine shuttle and the glyoxylate cycle, explains the absence of growth defects in either glyoxylate cycle, (*CIT2*), or carnitine shuttle mutants (*CAT2*). Indeed, only double mutants affected for both pathways are unable to grow on fatty acids as sole carbon source in the presence of low amounts of yeast extract (Van Roermund *et al.* 1995).

In *S. cerevisiae*, little is known about the function of carnitine besides its role in the transfer of acetyl-groups in the carnitine acetyltransferase system. We have developed a very specific strategy for the cloning of genes involved in carnitine-dependent activities in *S. cerevisiae*. We observed that a strain mutated in the *CIT2* gene, which encodes peroxisomal citrate synthetase, the first enzyme of the glyoxylate cycle, is dependent on carnitine for growth on oleic acid as sole carbon source. Through mutagenesis of this strain, we have isolated mutants affected in carnitine-dependent activities in the cell. The mutants fell into two phenotypical groups, (a) those that were unable to grow on media containing oleic acid as a sole carbon source supplemented with either yeast extract or carnitine alone, and (b) those which was not complemented by the addition of carnitine to the oleic acid media, but grew on the same media when complemented with low amounts of yeast extract. This suggests that yeast extract, which is used by other groups to further growth on oleic acid containing media, could contain compounds other than carnitine that overcome (or lessen) some of the defects associated with carnitine-dependent mutants. We have focused our research on the second type of mutant, since the genes affected in these strains must be specifically affected in carnitine-dependent activities alone. Seven independent strains with this phenotype were isolated. Through complementation of one of these mutants with a *S. cerevisiae* genomic library, we cloned the *YAT1* gene, encoding the presumptive outer-mitochondrial carnitine acetyltransferase (Schmalix and Bandlow 1993). No phenotype has previously been assigned to a mutant allele of this gene.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains and culture conditions.

Yeast strains used in this study are listed in **Table 3.1**. Yeast were either grown on rich YPD (1% yeast extract, 2% peptone, 2% glucose) or on minimal YNB media, containing 0.67% yeast nitrogen base (YNB) without amino acids (DIFCO) and 2% glucose. For phenotypical analysis, three different types of media with oleic acid as sole carbon source were used: (i) Oleic acid (OA) media, containing 0.67% yeast nitrogen base without amino

acids, 0.1% oleic acid, 0.4% Tween-40, and 0.5% of 0.5 M potasium phosphate buffer pH 6.0, (ii) Oleic acid/carnitine (OA+C) media, consisting of OA media supplemented with 10 mg/L of L-carnitine, and (iii) oleic acid/yeast extract media (OA+Y), containing 0.1% yeast extract instead of carnitine. Amino acids (20mg/L) were added to all media according to the specific requirements of the respective strains.

Table 3.1 Description of strains and plasmids used in this study

Strains and plasmids	Relevant genotype	Sources and references
Yeast strains:		
FY23	*MATa leu2 trp1 ura3*	Winston *et al.* 1995
FY23Δ*cit2*	*MATa leu2 ura3 Δcit2::TRP1*	This study
FY23Δ*yat1*	*MATa trp1 ura3 Δyat1::LEU2*	This study
FY23Δ*cit2*Δ*yat1*	*MATa ura3 Δcit2::TRP1Δyat1::LEU2*	This study
PSY142	*MATα leu2 lys2 ura3*	Liao *et al.* 1991
PSY142Δ*cit2*	*MATα leu2 lys2 Δcit2::URA3*	Liao *et al.* 1991
Plasmids:		
YEpLac112	2μ *TRP1*	Gietz and Sugino 1988
YCpLac33	CEN4 *URA3*	Gietz and Sugino 1988
YDp-L	*LEU2*	Berben *et al.* 1991
YDp-W	*TRP1*	Berben *et al.* 1991
YEpLac112-*CIT2*	*CEN4 TRP1 CIT2*	This study
YCpLac33-*CIT2*	*CEN4 URA3 CIT2*	This study
YCpLac33-*YAT1*	*CEN4 URA3 YAT1*	This study
pBluescript II SK+		Stratagene
pΔ*cit2*	Δ*cit2*::TRP1	This study
pΔ*yat1*	Δ*yat1*::LEU2	This study

3.2.2 DNA manipulation and construction of plasmids

All plasmids used in this study are listed in **Table 3.1**. Standard DNA techniques were carried out as described by Sambrook *et al.* (1989). Sequencing was carried out on an ABI automated sequencer. The *CIT2* gene was cloned by PCR using genomic DNA from strain FY23 as template. The forward primer 5-AGGTAAG CTTCTCGCTTAGGGTGCGG-3' and the reverse primer 5'-GAGGAATTCATCAGGTAAAGTTTCCTCGACC-3' were used. A 3443bp fragment, containing the *CIT2* gene with its native promoter and terminator sequences, was cloned into the multiple cloning site of YEpLac112 using the *Hind*III and *Eco*RI sites created by the two primers. The same *Eco*RI/*Hind*III fragment was also cloned into the multiple cloning site of the centromeric vector YCplac33. For disruption of *CIT2*,

the major part of the open reading frame was deleted by replacing the 1185 bp *Spe*I-*Bal*I fragment with a 823 bp fragment containing the *TRP1* gene, isolated from plasmid YDp-W The cloning construct and disruption cassette is represented in **figure 3.1**. A 1565 bp *Apa*I-*Stu*I fragment containing the disruption cassette was isolated and transformed into haploid wild-type FY23 cells. Trp^+ transformants were screened and the disruption of the chromosomal *CIT2* locus confirmed by analyzing the PCR product obtained on chromosomal DNA with the same primers used to amplify the gene.

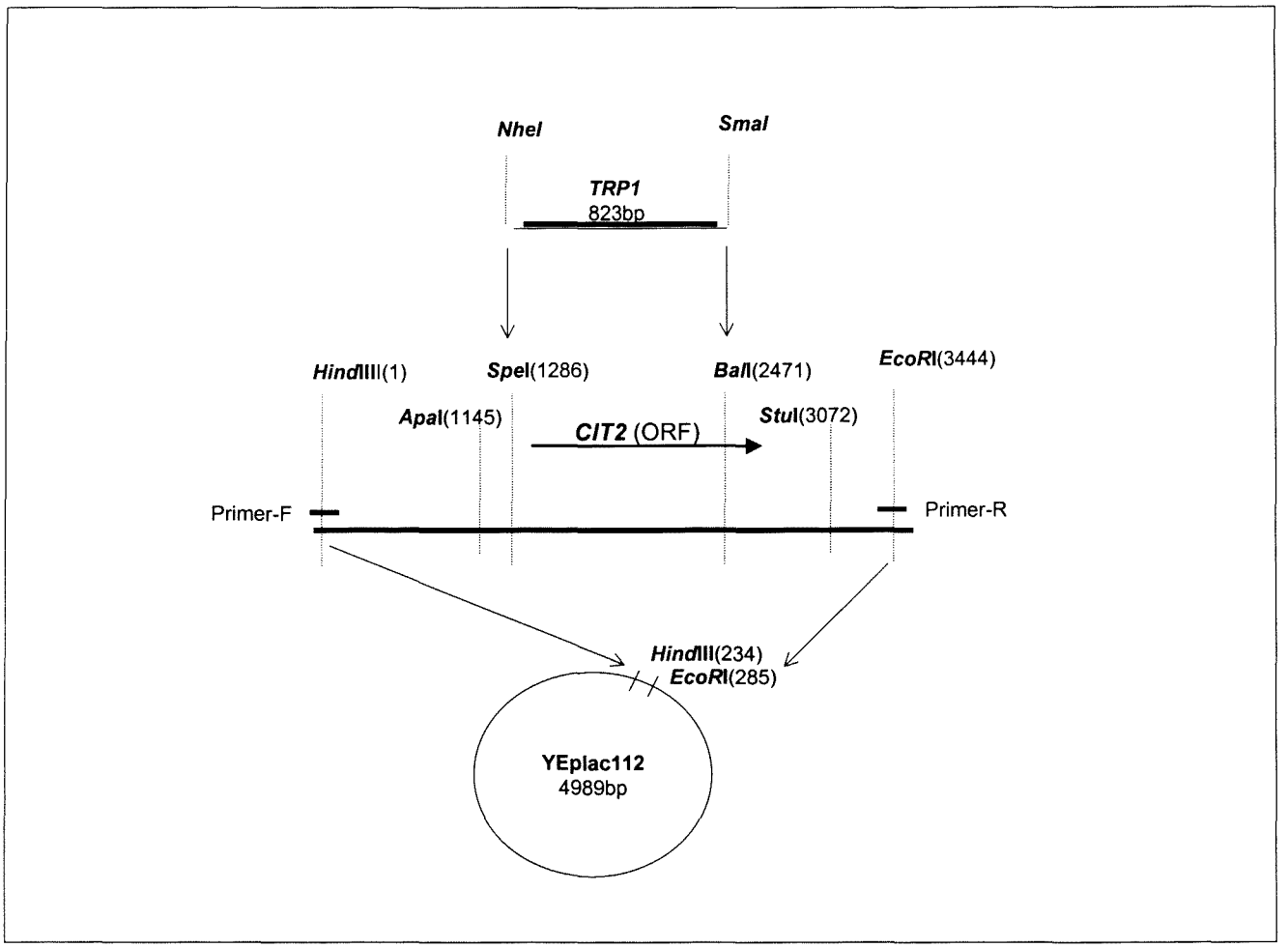

Figure 3.1 The cloning of *CIT2* and construction of the disruption cassette. The resriction sites *EcoR*I and *Hind*III was provided by the forward and reverse PCR primers and used to clone the 3443bp fragement containg *CIT2* into the YEplac112 plasmid. The *TRP1* gene was cloned into the *Spe*I and *Bal*I sites. The disruption construct was isolated by digesting with *Apa*I and *Stu*I.

3.2.3 Random mutagenesis of yeast strain

FY23Δ*cit2* cells were grown in YPD medium to an OD of 0.1 corresponding to $3x10^6$ cells/ml. From this culture, $4x10^8$ cells were spun down and washed twice with sterile water. The cells were suspended in 2 ml 0.1 M sodium phosphate buffer pH 7.0 and

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treated with 2% EMS for 1 hour at 30°C. The reaction was stopped by adding 5% (w/v) sodium thiosulphate. The survival rate, corresponding to the percentage of cells able to form colonies on rich YPD media after mutagenesis, was 25%.

3.2.4 Disruption of *YAT1*

A part of the ORF of *YAT1* was deleted by replacing the 819 bp *Sac*I fragment by cutting the YCpLac33-*YAT1* with *Sac*I followed by religation. The 1613 bp fragment containing the *LEU2* gene, isolated from plasmid YDp-L, was cloned into the *Bam*HI site of the open reading frame. The cloning construct and disruption cassette is represented in **figure 3.2**. A 2644 bp *Bal*I fragment containing the disruption casette was isolated and transformed into haploid wild-type FY23 and haploid FY23Δ*cit2*. Leu^+ transformants were screened and the genomic disruption confirmed by PCR using primers homologous to sequences upstream and downstream of the disrupted gene (5'-ATCAGCATCAGC ATCAGC-3') and (5'-AGAGGTAATCCAAACGACG-3').

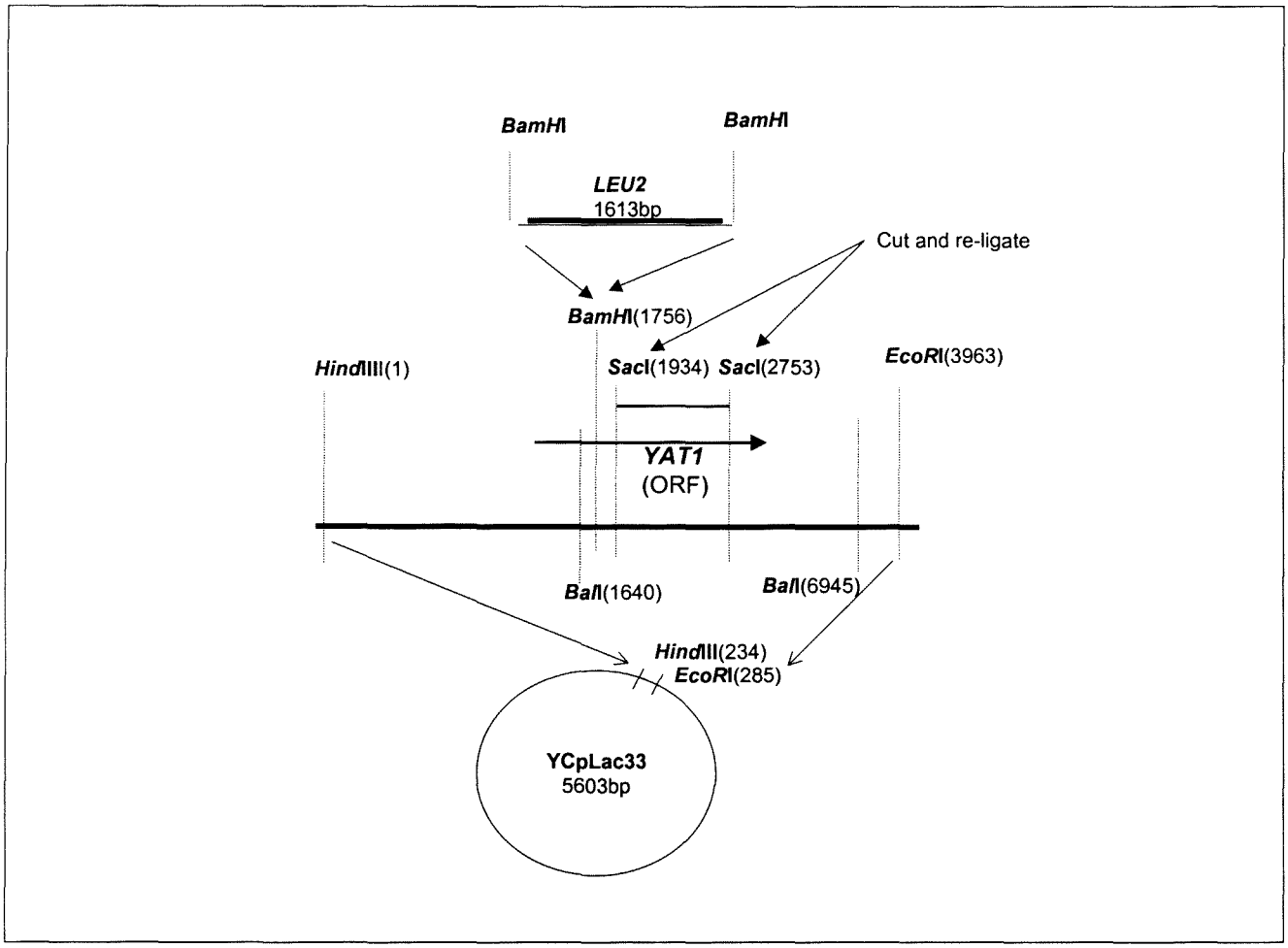

Fig. 3.2 The cloning of *YAT1* and the construction of the disruption cassette. A 3962bp fragment containing the *YATI* gene was cloned into the *Hind*III and *EcoR*I site of the YcpLac33 plasmid. The *Sac*I site was cut and re-ligated to remove a 819bp fragment of the open reading frame.

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3.3 RESULTS

3.3.1 Carnitine is not required for growth on oleic acid in a wild-type strain

Wild-type FY23 were grown on OA and OA+C liquid media and growth monitored by measuring the optical density of the culture. Growth on OA+C were slightly better than growth on OA (after 28 hours a difference in OD of 0.1 was observed). This slight difference was not sufficient for mutant selection. The relatively similar growth of the wild-type on both OA and OA+C indicates that either the wild-type cells could synthesize carnitine or that a by-pass exists. The latter is indeed the case, since Van Roermund *et al.* (1995) showed that the glyoxylate cycle was able to by-pass the requirement for Cat2p, the carnitine acetyltransferase essential in the carnitine shuttle.

3.3.2 A Δ*cit2* strain is dependent on carnitine for growth on oleic acid

In order to block the glyoxylate cycle we disrupted the the *CIT2* gene that encodes the peroxisomal citrate synthetase, a central enzyme of this cycle. The Δ*cit2* strains were tested on different growth media containing oleic acid as sole carbon source. The strains were unable to grow on OA, but grew on OA+C and OA+Y (**Tabel 3.2**). This result clearly demonstrates that the growth of this strain on oleic acid is carnitine-dependent. Furthermore, the results suggest that the ability of yeast extract to overcome the growth defect of a strain on oleic acid could be due to the presence of carnitine in yeast extract. The genetically unrelated PSY142Δ*cit2* strain showed the same phenotype as the FY23Δ*cit2* strain on OA and OA+C. This phenotype was also recently confirmed by Van Roermund *et al.* (1999) using a BJ1991Δ*cit2* strain. Taken together, the phenotypes of these strains clearly indicate that *S. cerevisiae* laboratory strains are unable to synthesize carnitine seeing that carnitine needs to be supplied in the medium in order for the strains to grow. The use of the two pathways needed for the utilization of acetyl-groups in the peroxisome of *S. cerevisiae* is represented in **Fig. 3.3**.

3.3.3 Selection of mutants affected for carnitine-dependent activities

In order to select mutants affected in genes encoding carnitine related activities, we took advantage of the carnitine-dependent phenotype of the FY23Δ*cit2* strain (**Table. 3.2**). After mutagenesis of this strain, cells were plated on YPD agar plates. After four days, colonies were washed off the YPD agar plates and replated on YNB-glucose agar plates. A total of 11 000 colonies were picked and streaked onto YNB-glucose plates in a grid-like arrangement at 110 colonies per plate and left to grow for three days. These plates were replica plated on OA+Y and OA+C and allowed to grow for 10 days (**Fig 3.4**). A total of 114 mutants defective for growth on oleic acid media, or oleic acid non-utilizing mutants

(*onu*), were isolated. Of these mutants, 90 (designated YHS1-YHS90) were unable to grow on OA+Y and OA+C media. In addition, an unexpected category of 24 mutants (designated YHSa-YHSx) did not grow on OA+C, but were able to grow on OA+Y. The phenotype of these 24 mutants suggests that yeast extract could contain additional growth factors, other than carnitine, that can relieve or lessen the effect of a *CIT2* disruption on oleic acid grown cells. The 24 strains were individually transformed with a centromeric plasmid containing the *CIT2* gene (YCpLac33-*CIT2*) in order to restore the by-pass glyoxylate cycle. Strains that grow on OA should be specifically affected for carnitine-dependent activities, since the fatty acid degradation-related functions, including β-oxidation or peroxisome biogenesis, should be unaffected. A total of seven mutants (YHSc; YHSd; YHSe; YHSi; YHSj; YHSs; YHSx) were isolated that could grow on OA after transformation with *CIT2*.

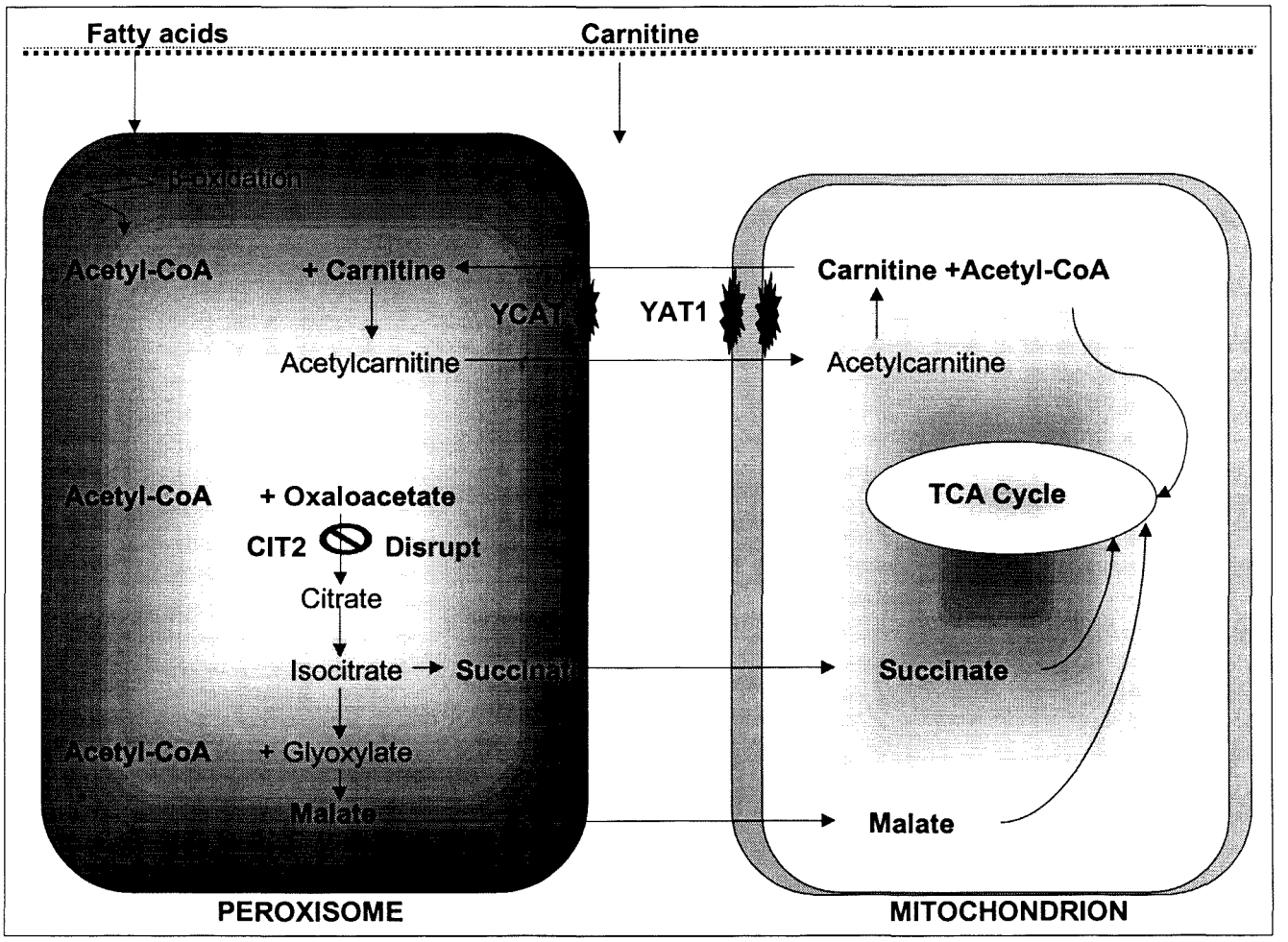

Fig. 3.3 The two pathways used in the utilization of acyl-groups in peroxisomes of *S. cerevisiae*. Some of the important enzymes are the glyoxylate enzyme citrate synthetase (*CIT2*), the carnitine acetyltransferase of the inner-mitochondrial and peroxisomal membrane (YCAT/*CAT2*) and the carnitine acetyltransferase of the outer-mitochondrial membrane (*YAT1*). Disruption of *CIT2* blocks the glyoxylate cycle, making the cell dependent on the carnitine shuttle.

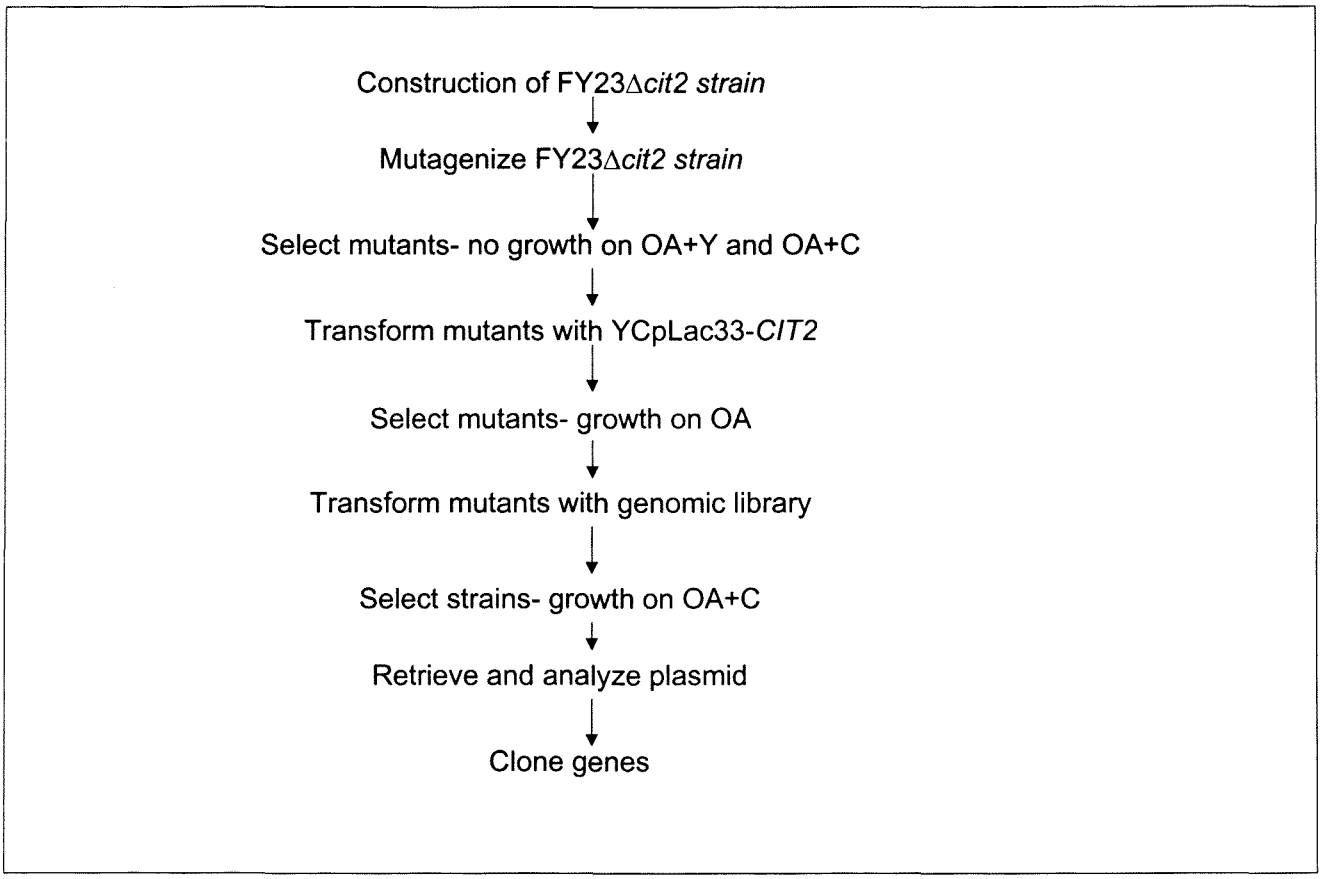

Fig. 3.4 Cloning strategy for the isolation of genes involved in carnitine-dependent activities

3.3.4 Cloning of *YAT1*

One of the seven isolated mutant strains, YHSx, was chosen at random for further work. The wild-type allele of the mutated gene in this mutant strain was cloned through complementation with a *S. cerevisiae* genomic library (ATCC 77162). The library was transformed into the mutant strain and colonies selected on YNB-glucose plates. A total of 6000 colonies was directly replica-plated onto OA+C media. After 10 days, only four colonies grew on this media. The four transforming plasmids were isolated from these strains. The complementing activity conferred by the plasmids was confirmed by re-transforming the isolated plasmids back into the mutant strain (YHSx). Restriction analysis revealed that three of the four isolated plasmids presented restriction patterns identical to the genomic *CIT2* locus. These plasmids also complemented the growth defect of the mutant strain (YHSx) on OA media, confirming that the insert contained the *CIT2* gene. The remaining plasmid showed an unrelated restriction pattern and was able to complement the mutant strain (YHSx) growth defect on OA+C media, but not on OA media. The genomic insert of 8.7kb was subcloned into pBluescript and both extremities were sequenced. Database searches (NCBI-BLAST) revealed that the insert contained a region located on chromosome I, and contained three complete open reading frames (**Fig.3.5**). One of these open reading frames corresponds to the *S. cerevisiae YAT1* which

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encodes the carnitine acetyltransferase of the outer-mitochondrial membrane. The *YAT1* with its native promotor was subcloned into the YCpLac33 multiple cloning site as a 3962bp *Hind*III/ *Eco*RI fragment, and complemented the mutant (x) phenotype on OA+C. Subclones of the other two open reading frames on the genomic fragment were unable to complement the phenotype. The *YAT1* plasmid was transformed into the remaining 6 mutant strains (YHSc; YHSd; YHSe; YHSi; YHSj; YHSs) and plated on OA+C to determine if other mutants were affected in the same gene. None of the mutant strains were complemented for growth on OA+C, indicating that the other mutants are mutated in other gene(s) required for carnitine-dependent activities.

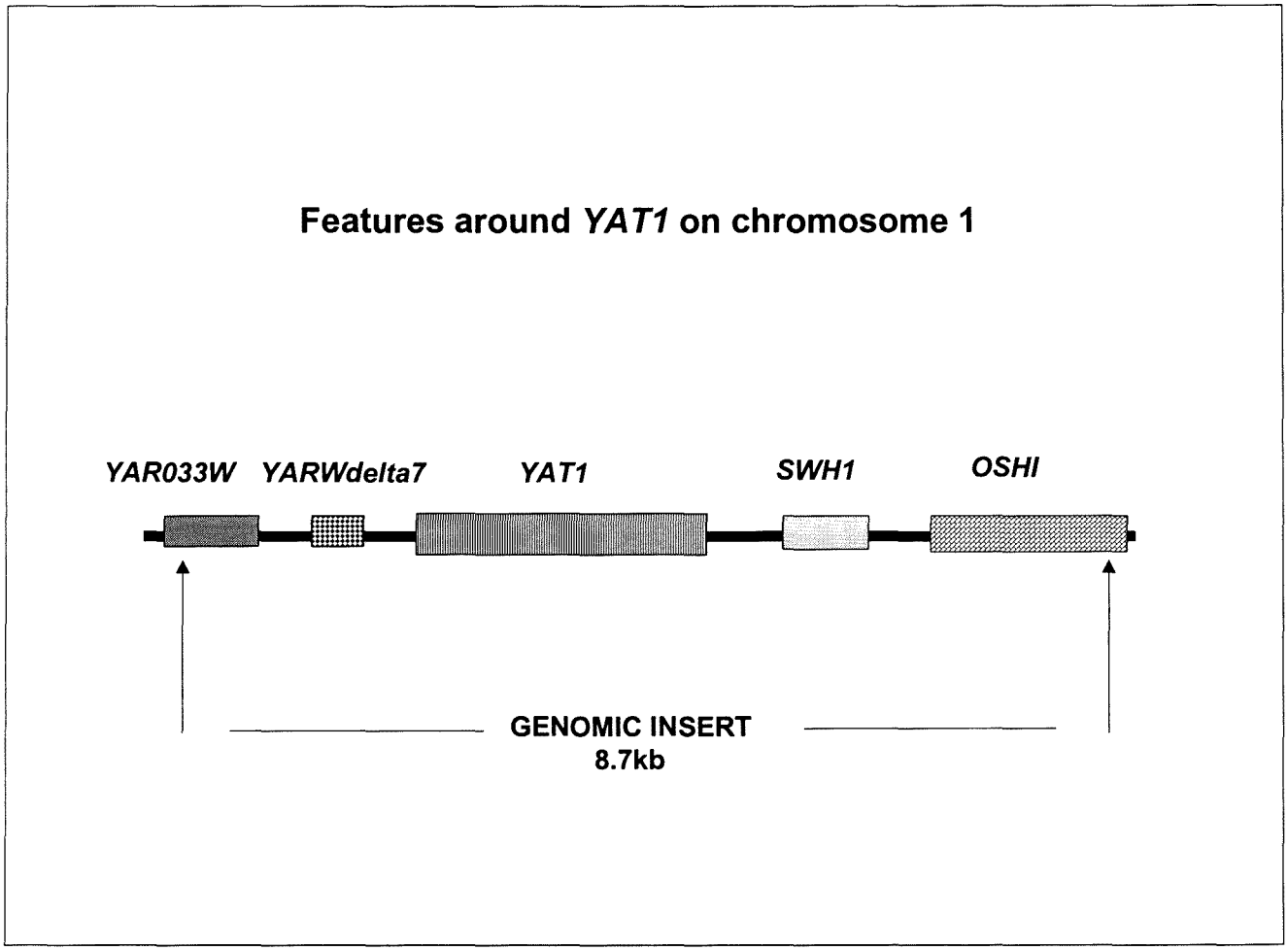

Fig. 3.5 The genes present on the genomic insert of the clone from mutant YHSx.

3.3.5 Disruption of *YAT1*

To verify that *YAT1* was the gene affected by the mutation introduced in the mutant strain (x), we disrupted *YAT1* in strains FY23 and FY23Δ*cit2*. As previously reported, the Δ*yat1* strain did not present any phenotypes and grew similar to the wild type strain on oleic acid media supplemented with yeast extract (OA+Y) (Schmalix and Bandlow 1993). In this

study, the Δ*cit2*/Δ*yat1* double mutant showed a phenotype similar to the isolated mutant YHSx, growing normally on OA+Y, but not showing any growth on OA+C (**Table 3.2**). The small amount (0.1%) of yeast extract present in the OA+Y media seems to allow for a by-pass for the *YAT1* function in the carnitine acetylcarnitine shuttle pathway. The data suggest that Yat1p is required for carnitine-dependent growth, but can possibly be made redundant by components other than carnitine present in yeast extract. This explains possible failures of other groups using OA+Y as standard media to identify *YAT1* as a gene involved in carnitine-dependent activities.

Table 3.2. The phenotype of strains used in this study. Phenotypes were monitored after replica-plating from minimal glucose media onto different oleic acid based media. These plates were incubating at 30°C for 14 days. (**+** indicates visible growth/ **-** indicates no visible growth)

Strain	OA +Y	OA +C	OA
FY23	+	+	+
FY23Δ*CIT2*	+	+	–
Mutant (Δ*CIT2*)	+	–	–
FY23Δ*CIT2*Δ*YAT1*	+	–	–
FY23Δ*YAT1*	+	+	+

3.4 DISCUSSION

In this study a strategy was devised for the cloning of genes specifically involved in L-carnitine-dependent activities in *S. cerevisiae*. This strategy was based on the knowledge that there are only two pathways in which peroxisomally produced acetyl-CoA can be further metabolized by the cell. One pathway is via the carnitine acetylcarnitine shuttle, while the other relies on the glyoxylate cycle that results in the net synthesis of the C_4 TCA intermediate succinate from acetyl-CoA. By blocking this second pathway through the disruption of the *CIT2* gene, we have created a strain that is dependent on the presence of carnitine in the growth media when fatty acids are used as sole carbon source. This implies that the strain is not able to synthesize its own carnitine. It is apparent that laboratory strains of *S. cerevisiae* do not synthesize carnitine. Recently, a similar cloning

strategy was devised by Van Roermund *et al.* (1999) and genes involved in carnitine-dependent activities were cloned.

Using the strategy in this study, a number of mutants unable to grow on oleic acid were isolated. Surprisingly, mutants were identified growing normally on oleic acid media containing yeast extract (OA+Y), but exhibiting no growth on oleic acid media containing carnitine (OA+C). The fact that carnitine is the only compound known to be involved in the transfer of acyl-groups from the peroxisome to the mitochondria makes this an interesting result. The *YAT1* gene, which encodes a carnitine acetyltransferase of the outer-mitochondrial membrane, was identified as the gene mutated in one of our seven mutants. This result confirms that this strategy targeted carnitine-dependent activities efficiently, suggesting that the other six mutant strains with similar phenotypes will allow for the identification of additional genes required for the carnitine-dependent activities. These mutants are not affected in *YAT1* activities, since this gene was unable to complement any of the six mutant phenotypes. Disruption of *YAT1* confirmed the phenotype of the mutant strain initially selected, indicating that the primary mutation displayed a null mutant phenotype. It is surprising that a Δ*yat1* mutation in a Δ*cit2* background results in a strain growing normally on oleic acid media containing yeast extract (OA+Y). This result indicates that possible growth factors other than carnitine influence the requirements for an efficient carnitine acetylcarnitine shuttle system. The data clearly showed that in the carnitine-dependent strain, *YAT1* is absolutely required for growth on oleic acid when carnitine is the only additional co-factor added.

The sole role attributed to L-carnitine in *S. cerevisiae* is to act as a carrier molecule for the transfer of acyl-groups from the peroxisomes to the mitochondria via the carnitine acetylcarnitine shuttle. To date, only four genes have been identified that are implicated in being involved in carnitine-dependent activities in this organism: (i) *CAT2*, encoding the carnitine acetyltransferase of the peroxisomal and inner-mitochondrial membrane (Kispal *et al.* 1993; Elgersma *et al.* 1995); (ii) *YAT1*, encoding the carnitine acetyltransferase of the outer-mitochondrial membrane (Schmalix and Bandlow 1993); (iii) *AGP2*, encoding the plasma membrane carnitine transporter (Van Roermund *et al.* 1999) and (iv) *CAC*, the carnitine acetylcarnitine translocase of the inner-mitochondrial membrane (Van Roermund *et al.* 1999). The selection screen used by van Roermund *et al.* (1999), resulted in the isolation of three of these genes, namely *CAT2*, *AGP2* and *CAC*. We showed that their failure to identify *YAT1*, and possibly other genes involved in carnitine-dependent activities, is due to the selection of mutants on oleic acid supplemented with yeast extract (OA+Y), and not on oleic acid supplemented with carnitine (OA+C) as was done in this study.

Cloning the genes responsible for the phenotypes of the remaining six mutants will provide clearer answers to some of the questions, and, hopefully cast some light on the cellular

functions of carnitine. The screening strategy used in this study was very specific for carnitine and only genes affected in carnitine-dependent activities should be isolated if this screen is used. As shown here, a screening method based on media containing oleic acid supplemented with yeast extract, will not result in the identification of all of the genes affected in carnitine-dependent activities.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

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GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION AND OTHER PERSPECTIVES

In the last few decades, yeast, especially *S. cerevisiae*, has been used as a model organism in the study of many processes that take place in the eukaryotic cell. Yeast has been used with particular success in the study of peroxisome biogenesis (reviewed in Van der Klei and Veenhuis 1997). It was the aim of this thesis to highlight the importance of the peroxisome and to report on the progress made in unraveling the biogenesis of this interesting organelle. An effort has also been made to explain the different functions of the peroxisome and to explain the metabolic interaction of the peroxisome with the cytosol and other organelles. One of the most important metabolic interactions is the exchange of activated acetyl groups, produced in the peroxisome through the β-oxidation of fatty acids, with the cytosol and the mitochondrion. This exchange is mediated by the carnitine acetylcarnitine shuttle, which itself depends on carnitine. The experimental section of this thesis focused on the identification of molecular components involved in carnitine-dependent activities, which would include components of the carnitine acetylcarnitine shuttle.

L-Carnitine plays an important role in the eukaryotic cell. In yeast, carnitine plays a role in the transfer of activated acetyl groups from the peroxisome to the mitochondria (reviewed in Bieber 1988). Its function is therefore closely linked to the peroxisome. Little is known about whether other roles for carnitine exist in yeast, as is the case for higher eukaryotes. In mammalian cells, other functions like the regulation of the acyl-CoA/ CoA-SH ratio, the elimination of selective acyl residues (detoxification) and carnitine acting as a reservoir of activated acetyl units, have been described (reviewed in Bieber 1988).

The focus of the literature review was therefore on the peroxisome, a organelle that plays a pivotal role in our mutant selection strategy. Peroxisomes are small, single membrane bound organelles approximately 0.2-1μm in diameter and present in most eukaryotic cells. The name peroxisome was derived from the fact that these organelles produce hydrogen peroxide as a by-product of their metabolic activity (de Duve and Bauduin 1966). The peroxisomes perform a large range of metabolic roles in eukaryotic cells, some of which are common to all organisms like β-oxidation of fatty acids, whereas others, like photorespiration in plant leaves and ether-lipid synthesis in mammalian cells, are specific to peroxisomes of these organisms (reviewed in Van den Bosch *et al.* 1992).

The study of peroxisomes in yeast began with the discovery of their presence in *S. cerevisiae* (Avers and Federman 1968). Since then, a lot of research has been

conducted in yeast regarding the function and biogenesis of peroxisomes. The first few sections of Chapter 2 focus on the structure and some of the functions of peroxisomes, which include peroxisomal respiration, β-oxidation of fatty acids and the glyoxylate cycle (reviewed in Van den Bosch *et al.* 1992). These functions are required when yeast are grown on fatty acids. The second part of Chapter 2 focuses on peroxisome biogenesis. This includes the isolation of *pex* mutants and the identification of components involved in peroxisome biogenesis. Particular attention is given to the import of peroxisomal matrix and membrane proteins and the possible mechanisms of actions are discussed. Most of the *PEX* genes known are discussed in this section. The last part of Chapter 2 focuses on the import of folded proteins into peroxisomes and the degradation of peroxisomes.

Cleverly designed selection methods used in yeast have been at the heart of the identification of all the *PEX* genes identified up to date. Initially, selection methods in *S. cerevisiae* were based on a negative screen identifying mutants unable to grow on oleic acid (Erdmann *et al.* 1989). However, most of these mutants were not peroxisome biogenesis mutants. Subsequently, more specific selection methods were developed using for example the toxicity of hydrogen peroxide and the neutralizing effect of bleomycin on the phleomycin ligand (Van der Leij *et al.* 1992; Elgersma *et al.* 1993). In the experimental section of this thesis, possible peroxisome biogenesis mutants were also isolated through selecting for growth defects on oleic acid. However, the peroxisome biogenesis mutants were discarded in a step that exploited the existence of the glyoxylate cycle in yeast. The manipulation of the glyoxylate cycle in the yeast peroxisome has been the key element in developing a carnitine-dependent strain and in selecting for mutants affected in carnitine-dependent activities. Through disruption of the citrate synthetase gene, *CIT2*, we blocked one of the two pathways used for the transfer of activated acyl-groups out of the peroxisome (Van Roermund et al. 1995). In this way, we could focus on the other pathway used for the transfer of activated acyl-groups out of the peroxisome, namely the carnitine shuttle.

Other mutations that would have been selected for in the first selection step are mutations in the β-oxidation cycle and import of fatty acids into the cell and peroxisome. In yeast, the peroxisome is the sole site for β-oxidation (reviewed in Kunau *et al.* 1988). Mutants unable to grow on oleic acid were used to identify some of the enzymes in *S. cerevisiae* involved in the β-oxidation cycle (Dmochowska *et al.* 1990; Hiltunen *et al.* 1992). We were interested in mutants that had a functional β-oxidation cycle so that fatty acids could be oxidized to acetyl-CoA in the peroxisomes. The transformation with *CIT2* allowed us to select only those mutants that were functional in their β-oxidation cycle and fatty acid import.

The selection strategy proved to be accurate when the *YAT1* gene, mutated in one of the seven mutants, was cloned. *YAT1* is the carnitine acetylcarnitine transferase of the outer

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mitochondrial membrane (Schmalix and Bandlow 1993). Cloning of this gene proved that our selection strategy is specific for the isolation of genes involved in carnitine-dependent activities. Theoretically, all the other mutants should therefore also affect genes involved in carnitine-dependent activities. Disruption of the *YAT1* gene showed some novel phenotypes not yet described. Its specificity towards carnitine is a point of interest and the ability of minimal amounts of yeast extract to overcome the phenotype of a disrupted *YAT1* gene in a strain without the glyoxylate cycle, are novel findings to further investigate.

Identification of other genes using this selection strategy should produce some novel components and data regarding the role of carnitine in *S. cerevisiae*. Our goal is to clarify the exact role of carnitine in the cell and to extrapolate that to human systems where diseases like primary carnitine dificiency have a devastating impact on the well-being of patients. Future work in this field would not only prove to be useful in understanding the yeast *S. cerevisiae*, but also in improving the quality of life for humans.

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